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MUTATION CONFERRING STREPTOMYCIN RESISTANCE IN XANTHOMONAS PHASEOLI[1]

C. QUADLING

Abstract

Spontaneous mutations conferring resistance to the action of 500 to 2000 μg/ml streptomycin occur at an over-all rate of about 3×10^{-10} per bacterium per generation in *Xanthomonas phaseoli*. Some mutants will not grow in the absence of streptomycin; others grow both in the presence and in the absence of the drug. In most cases resistance to high concentrations of streptomycin does not affect the virulence of this organism for bean plants.

Introduction

There is a large body of literature dealing with the subject of drug resistance in bacteria (20). Naturally, much experimental work has been done on bacteria of medical or quasi medical interest, such as *Aerobacter aerogenes*, *Diplococcus pneumoniae*, *Escherichia coli*, and *Salmonella*. In a number of cases the character of "drug resistance" has been used as a convenient research tool in genetical studies on bacteria.

Although of fundamental importance, data on the genetic stability of bacteria of agricultural interest is sparse (21). This paper reports one aspect of a series of investigations into the variability of a phytopathogen, *Xanthomonas phaseoli* (Smith) Dowson, that causes "bacterial blight" of beans. It describes spontaneous mutation conferring resistance to the lethal effects of high concentrations of streptomycin. The work was undertaken for two reasons: to provide appropriate systems which could be used to test for possible mutagenic effects of agricultural chemicals and to yield information on the genetics of the plant pathogen. The rate of mutation was determined and some properties of resistant organisms investigated.

Materials and Methods

Streptomycin, calcium chloride complex (Merck), was used at 500–1000 μg/ml for selective purposes. Difco media were used throughout: for solid medium Antibiotic Medium No. 2 (Penassay agar) with addition of dipotassium phosphate 3.68 g/l. and monopotassium phosphate 1.32 g/l.; for liquid

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medium Antibiotic Medium No. 3 (Penassay broth) was used. For convenience these media will be referred to as nutrient agar and nutrient broth respectively.

All work was carried out with strain XP8 of *Xanthomonas phaseoli* (Smith) Dowson, originally obtained from the stock culture collection of the Plant Research Institute, Canada Department of Agriculture, or with substrains of XP8 as explained below. Stock cultures were derived from single colonies. These were allotted postscript numbers, e.g., XP8-1, XP8-2 (Table I). At first, stock cultures were maintained on chalk–glucose–yeast agar slopes (5), or on nutrient agar slopes, stored at 4° C and subcultured monthly. It was later found convenient to store cultures in frozen nutrient broth containing 15% (v/v) glycerol (9), held at −20° C. No adverse genetic effects resulting from such treatment were detected. Where necessary the identity of cultures was confirmed by patch testing with phage PXP8 (11) specific for *X. phaseoli*.

As far as possible all experiments were carried out with bacteria in the logarithmic phase of growth. Cultures for use in experiments were derived by streaking stock cultures onto nutrient agar; after incubation one or a small number of single colonies were transferred to 50 ml nutrient broth in a 300-ml Belco "nephelo-culture" flask and incubated with shaking at 26° C. Under these conditions XP8 has a mean generation time of about 2 hours. Turbidity measurements were made with a Klett colorimeter, using a green filter. Viable counts were carried out by making pour-plates of aliquots of suitable dilutions or by the drop method of Miles and Misra (14) except that a platinum-tipped dropping pipette was employed in place of standard Pasteur pipettes.

Ultraviolet irradiation of cultures was carried out for 2 to 3 minutes at a distance of 30 cm from a "Mineralight" source (Ultra-violet Products Inc., South Pasadena, California) equipped with a 2537 Å filter. Bacterial suspensions were first spread on the surface of nutrient agar; 90% to 99.9% were killed by this ultraviolet treatment.

When cultures were grown on nutrient agar, streptomycin-resistant mutants were selected by methods similar to those of Newcombe (18) in which streptomycin was added as an overlay of sterile nutrient agar. In the present work

TABLE I

Strains referred to in the text

Strain No.	Origin	Relevant character
XP8-1	Single colony pick	"Wild type" *X. phaseoli* (prototrophic)
XP8-24	From XP8-1 by ultraviolet irradiation	Partial auxotroph, responds to nicotinamide
XP8-26	From XP8-1 by ultraviolet irradiation	Partial auxotroph, responds to homocysteine
XP8-47	From XP8-1 by streptomycin selection	Streptomycin indifferent
XP8-52	From XP8-24 by streptomycin selection	Streptomycin indifferent, auxotroph, responds to nicotinamide
XP8-60	From XP8-1 by ultraviolet irradiation and streptomycin selection	Streptomycin dependent
XP8-105	From XP8-26 by streptomycin selection	Partial auxotroph, partially dependent on streptomycin

bacteria were grown on the surface of 15 ml of nutrient agar and the overlay consisted of 5 ml nutrient agar with 10^4 μg/ml streptomycin.

Strains of *X. phaseoli* were tested for virulence for bean plants by the inoculation method of Dye (6) and also by needle puncture at the primary leaf node. Young plants of variety "Pacer" or of "Clipper" grown under greenhouse conditions were used in this work. Both varieties were equally susceptible to "wild type" *X. phaseoli* (XP8).

Results

Effects of Low Concentrations of Streptomycin

When suspensions of *X. phaseoli* (XP8-1) were added to nutrient agar containing low concentrations of streptomycin, the number of bacteria which formed colonies varied with the concentration of streptomycin and the numbers of bacteria inoculated. Results are given in Table II of an experiment in which aliquots of a suitably diluted, logarithmic-phase culture of XP8-1 were added to melted streptomycin-containing nutrient agar which was then allowed to set in Petri dishes. We conclude that this "wild type" strain of *X. phaseoli* is sensitive to the killing action of low concentrations of streptomycin and that most of the bacteria are killed at concentrations between 2 and 8 μg/ml.

When aliquots of dilutions of XP8-1 were spread on the surface of nutrient agar, rather than mixed with melted medium containing low concentrations of streptomycin, the proportion of bacteria surviving was larger and considerable heterogeniety apropos of colonial morphology both within and between colonies was noticed amongst survivors. Presumably threshold concentrations of streptomycin exist below which sensitive cells have a high probability of surviving. However, partial inhibition by the drug may favor the establishment of faster growing variants. Hurwitz and Rosano (10) reported the reversal of streptomycin inactivation of *Escherichia coli* by phosphate; these authors used low concentrations of streptomycin (10 μg/ml) and found the greatest effect of phosphate occurred when cultures were aerated. The greater survival obtained in surface platings suggests that reversal of streptomycin action occurs in *X. phaseoli*. The effects of low concentrations of streptomycin were not investigated further.

TABLE II

Counts of colonies of *X. phaseoli* (XP8-1) growing in nutrient agar plates containing low concentrations of streptomycin*

Dilution	Approx. no. bacteria plated	No. colonies† formed in presence of streptomycin (μg/ml)						
		0	0.5	1	2	3	4	8
10^{-6}	50	48	55	32	42	1	0	0
10^{-5}	500	+‡	+	+	14	0	0	0
10^{-4}	5000	+	+	+	+	89	0	0

*Aliquots (0.1 ml) of dilutions of a culture containing about 5×10^8 sensitive bacteria/ml were added to each plate.
†Means of three plates.
‡More than 100 colonies per plate.

Effects of High Concentrations of Streptomycin

When large populations of *X. phaseoli* (XP8-1) were exposed to the action of high concentrations of streptomycin (500–2000 μg/ml) in nutrient agar medium only a very small minority of the treated bacteria produced colonies. Seven additional unrelated "wild-type" cultures of *X. phaseoli* and one strain of each of six other *Xanthomonas* species were tested for sensitivity to high concentrations of streptomycin; all were sensitive. In a typical experiment in which replicate cultures of XP8-1 were treated with streptomycin a total of 138 resistant colonies grew, representing a proportion of approximately one resistant organism in each 4×10^8 sensitive bacteria tested. In this paper the term "resistance" is used in a general sense to describe the observed property of organisms which survive exposure to streptomycin. However, as is well known (18, 22, 23) different types of resistant organisms occur. In the present work at least three types of mutant resistant to high concentrations of streptomycin were found. Most resistant organisms derived from XP8-1 by single-step selection grew both in the presence and in the absence of the drug; these mutants were presumably analogous to the fast-growing "streptomycin-indifferent" mutants described in *Salmonella* by Watanabe and Watanabe (22, 23) and this term will therefore be used to describe them.

In some circumstances resistant mutants which grew only in the presence of streptomycin were isolated. Such streptomycin-dependent mutants were rare in XP8-1; in one series of experiments a total of 78 resistant colonies representing that number of separate mutational events were tested; none was dependent. However, following ultraviolet irradiation of XP8-1 (approximately 90% bacteria killed) and delayed streptomycin selection about 30% of the resistant colonies found were totally dependent.

A third class consisting of partially dependent mutants occurred spontaneously in a particular substrain of *X. phaseoli* (XP8-26). This semiauxotrophic strain did not give rise to indifferent mutants when plated on streptomycin nutrient agar. The resistant colonies were partially dependent and grew at about normal rates in the presence of the drug but very slowly in its absence. Partially dependent mutants were not found in experiments with other strains derived from XP8-1 but no special search was made. Such mutants have been described in *E. coli* (18).

Spontaneous Nature of Mutation to Streptomycin Resistance

Lederberg and Lederberg (12) described the replica-plating technique of indirect selection which enables the demonstration of the fact that resistant mutants exist in a culture prior to exposure to a selective agent. Streptomycin-resistant mutants have been isolated from XP8 by means of this technique. We conclude, in accordance with the results obtained with other organisms (12, 2), that exposure to streptomycin is not a necessary condition for the occurrence of mutation conferring resistance to this drug.

Estimation of Mutation Rate

An estimate has been made of the rate at which mutation occurs, to confer resistance to high concentrations of streptomycin, by means of experiments modelled on the "*Po*" method of Luria and Delbrück (13) in which 30 to 50 replicate cultures were tested in their entirety for the presence of resistant mutants. Mutation rates can be calculated from the proportion of such cultures which contain no mutants and the average number of bacteria per culture at the time of testing (13, 15). Replicate cultures were grown either in 10-ml volumes of nutrient broth in 50-ml Erlenmeyer flasks incubated with shaking at 26° or on the surface of nutrient agar in Petri dishes.

In a typical experiment a set of 50 preincubated nutrient agar plates were inoculated by spreading about 10^6 organisms contained in 0.5 ml of a logarithmic-phase culture of XP8-1. A small number of plates were at once tested for the presence of streptomycin-resistant mutants in the inoculum by the addition of an overlay of streptomycin-containing nutrient agar. The remainder of the plates were incubated at 26° C for about 15 hours to allow an approximate 1000-fold increase in numbers of bacteria. Independent viable counts were carried out on bacteria from three or more plates: cells were washed off the agar surfaces by transfer of the entire contents of the Petri dish to a jar of cold (4° C) sterile broth, followed by 30-minute agitation on a shaker. Plate counts were made from suitable dilutions of the resulting suspension. Reconstruction experiments showed that this method gave a satisfactory (greater than 90%) recovery of organisms. Streptomycin-containing nutrient agar (10^4 μg/ml) was added as an overlay to the remainder of the plates to kill sensitive bacteria. After a further 5 to 7 days' incubation the streptomycin-treated plates were examined and the presence or absence of resistant colonies recorded. When it was desired to count numbers of resistant colonies, plates were scrubbed with a glass spreader and sterile broth prior to the addition of the overlay. Some observed distributions of numbers of resistant colonies amongst replicate plate cultures are given in Table III. The identity of drug-resistant colonies was confirmed by the results of subsequent picking and cross-streaking with phage (11) for *X. phaseoli*. In this series of experiments

TABLE III

Distribution of numbers of streptomycin-resistant (1000 μg/ml) colonies amongst series of replicate plate cultures of *X. phaseoli* (XP8-1)*

Number of colonies per plate																
0	1	2	3	4	5	6	7	8	9	10	11	13	15	20	22	25
Frequency																
8†	3	3	4	1	1	2	3	1	1	0	0	1	0	1	0	1
21	0	4	2	1	1	1	2	0	0	0	0	0	1	0	1	0
17	4	3	4	3	1	1	1	0	0	0	1	0	0	0	0	0

*Plates scrubbed with glass spreader to redistribute resistant bacteria prior to addition of overlay containing streptomycin.
†Experiment 1 of Table IV.

TABLE IV

Estimation of rate of mutation conferring streptomycin resistance in *X. phaseoli* (XP8-1)*

Expt. No.	Viable counts† per culture		No. cultures:		Mean no. mutational events per culture‡	Mutation rate§
	Inoculum $\times 10^{-6}$	Final $\times 10^{-8}$	With mutants	Without mutants		$\times 10^{10}$
1	1.90	18.10	22	8	1.31	5.02
2	1.33	6.13	17	33	0.40	4.74
3	1.66	5.05	16	66	0.21	2.88
4	1.97	28.20	45	48	0.65	1.60
5	3.60	60.00	37	9	1.61	1.86
6	0.25	27.50	30	17	1.02	2.57
7	2.80	19.50	19	26	0.54	1.93
8	3.30	22.00	39	13	1.39	4.38
9	0.40	3.56	4	18	0.20	3.89
10	1.22	7.15	10	25	0.34	3.29
Mean						3.24

*Experiments involving tests on series of replicate cultures for the presence of mutants. Replicate cultures were grown in flasks in Nos. 3 to 8 inclusive; plate cultures were used in the remainder.
†Means of three or more independent plate counts.
‡Calculated by the Poisson formula: $Po = e^{-x}$ where Po is the proportion of cultures without mutants.
§Calculated by Method II of Catcheside (1).

no distinction was made between streptomycin-indifferent and streptomycin-dependent colonies; all were classed together for counting purposes. The above experimental procedure was appropriately modified in experiments in which liquid media, rather than solid, were used for the growth of the replicate cultures. By means of appropriate calculations, e.g., formula 1 of Newcombe (15) or method II of Catcheside (1), an estimate of the over-all mutation rate to streptomycin resistance can be obtained from these data. Results for a number of such experiments are listed in Table IV.

Properties of Resistant Mutants

As mentioned above, at least three types of mutant occurred. Growth rates were determined for some isolates. A streptomycin-indifferent mutant (XP8-47) obtained by single-step selection with 500 μg/ml streptomycin grew in nutrient broth at 26° C with a mean doubling time (turbidity measurements) of about 2 hours. Growth curves, obtained in the presence or absence of streptomycin, were indistinguishable. A totally dependent mutant (XP8-60) in the presence of 500 μg/ml streptomycin at 26° C had a mean doubling time of about 4 hours, judged by turbidity measurements. When washed twice and resuspended in streptomycin-free nutrient broth growth continued normally for about one mean generation time and then at a lesser and decreasing rate for an additional 30 hours in which time a fourfold increase in turbidity occurred. No colonies were formed when such cultures were plated on nutrient agar, with the exception of colonies produced by rare (about 2 in 10^7 streptomycin-grown cells plated) back mutants which grew in the absence of streptomycin. Of 52 such non-dependent colonies tested only two were still streptomycin-resistant, the remainder were sensitive to the drug.

To investigate the possibility that a proportion of resistant mutants were killed by the high concentrations of streptomycin used for selection, some reconstruction experiments were performed. Plate counts of aliquots of a culture of streptomycin-indifferent mutant (XP8-47) were made on nutrient agar containing, respectively, none, 10^2, 10^3, 10^4, and 10^5 μg/ml streptomycin. At 10^5 μg/ml an approximately 20% reduction was observed in viable count; no effect was found at the other concentrations tested. In another experiment, plate counts were carried out in the presence of 5×10^3 μg/ml streptomycin and approximately 10^9 sensitive bacteria per plate. The expected number of added, resistant bacteria were recovered as colonies after incubation. We conclude that the concentrations of streptomycin employed in experiments were not lethal to the resistant organisms and that the initial presence of sensitive bacteria did not introduce errors into the counts of resistant mutants.

Effects of Mutation on Virulence

In different examples from animal pathology, streptomycin resistance has been reported to leave unchanged (7) or to reduce (7, 24) bacterial virulence. Because streptomycin has been used to control plant disease (25) it was of interest to see what effect, if any, such mutation had on virulence of *Xanthomonas*.

The virulence of *X. phaseoli* strains for beans (variety Pacer) was tested by the method of Dye (6), in which drops of culture are placed on the unopened buds which will give rise to the first trifoliate leaf of each plant. Five to 10 replicates were made of each test and all experiments were repeated at least once.

In the absence of a satisfactory objective test of virulence, disease symptoms (leaf spots, lesions, wilting) were rated on inspection after 10 and 30 days as severe, moderate, or absent. XP8-47, a streptomycin-indifferent mutant of wild type XP8-1, produced severe symptoms of bacterial blight indistinguishable from those of the parent strain. Plants inoculated with the totally dependent XP8-60 showed no symptoms of disease, as might be expected. The effect on virulence of acquisition of streptomycin resistance was tested in combination with a number of single mutations involving nutritional deficiencies. In only one case was streptomycin resistance associated with an appreciable change in virulence. An ultraviolet-induced mutant XP8-24, which required nicotinamide but grew very slowly in its absence, when made streptomycin indifferent by selection became completely dependent on nicotinamide for growth. At the same time the effect of this strain on the test plants changed from one of moderate virulence to avirulence. Presumably this was due to an associated effect of the streptomycin character on nicotinamide metabolism (reinforcing the already present defect) rather than of streptomycin resistance per se. Watanabe and Watanabe (23) found streptomycin-resistant mutants in *Salmonella* which required both thiamine and nicotinic acid for growth. Garber (8) has pointed out that bacteria unable to grow in the nutritional environment provided by a host will be unable to cause disease symptoms.

Discussion

The results reported here demonstrate that clones with the heritable character of indifference to the action of high concentrations of streptomycin occur spontaneously in sensitive cultures of *X. phaseoli.* Streptomycin-indifferent clones occurring in *X. phaseoli* are presumably analogous to the fast-growing indifferent mutants described in *Salmonella* by Watanabe and Watanabe (22). As reported for *E. coli* (18) streptomycin-dependent clones occur in *Xanthomonas.* Suggestive but not conclusive evidence has been obtained for the existence of unstable clones with lesser degrees of resistance. Watanabe and Watanabe (22) report on such mutants in *Salmonella* and comment on the genetic instability of at least some of them.

The conclusion that the change from sensitive to streptomycin indifferent or dependent is mutational and spontaneous in nature rests on two complementary lines of evidence. The indirect selection technique of Lederberg and Lederberg (12) by which streptomycin-resistant clones can be isolated without direct contact with the drug is of itself (2) an adequate test of the undirected nature of mutation to resistance. The distribution of numbers of resistant colonies (Table III) obtained in experiments modelled on the "*Po*" method of Luria and Delbrück (13) is compatible with expectation on the hypothesis of random mutation giving rise to a Poissonian distribution of mutational events, but not of mutant organisms. Reasonably reproducible results (Table IV) were obtained from experiment to experiment, but no high degree of accuracy can be claimed for the calculated mutation rates because segregation (19) and phenotypic lags have not been taken into account. If present, assuming that streptomycin resistance is a recessive characteristic, such lag would result in the calculated mutation rates being lower than those obtaining in reality.

Another source of error requires comment. Table IV reports a series of results obtained with about 500 cultures grown from a total inoculum of approximately 10^9 bacteria. Because an average of 2.5 resistant bacteria are present in cultures containing 10^9 organisms it is likely that this number of cultures contained resistant bacteria at the start of incubation. This could result in a spurious increase in the proportion of cultures found to contain mutants, at the end of the experiment. Such errors could result in the calculated mutation rate being greater than the true value and would be least serious in experiments in which the numbers of cultures with and without mutants were near equal. For example, if in experiment 4 (Table IV) one culture were assumed to be falsely recorded as positive then the corrected mutation rate would be 1.57×10^{-10} instead of the value shown.

The "nuclear" rather than cytoplasmic nature of mutation leading to streptomycin indifference has been demonstrated, amongst others, by Newcombe and Nyholm (12) and Watanabe and Watanabe (22). Evidence that this is also true of *Xanthomonas* is provided by the report of Corey and Starr (3) that desoxyribonucleic acid (DNA) mediated transformation to streptomycin resistance occurs in this genus. Unfortunately, efforts to demonstrate genetic exchange in our strain (XP8) by means of DNA transformation (Taka-

hashi, personal communication) or by whole cell recombination (Quadling, unpublished) have been unsuccessful. Because of this present lack of genetic analysis it is not possible to draw conclusions about the number of loci concerned in the present work. However, the rates of mutation found here are very similar to those found in *E. coli* (16) and because it appears that a single locus is concerned with streptomycin indifference (4, 17) in that genus it seems very likely that a similar locus is present in *Xanthomonas*.

One might expect variants with slight degrees of resistance to emerge after the application of small doses of streptomycin to infected plants. Unfortunately such variants were not tested for virulence in the present work. Whilst the results obtained with streptomycin-indifferent mutants suggest that such resistance does not appreciably alter virulence, it seems likely that mutants with reduced growth rates will be less virulent than those with growth rates similar to those of the "wild type" pathogen. This view is supported by the fact that the partially dependent mutant XP8-105 (Table I), which grew very slowly in the absence of streptomycin, produced no disease symptoms in inoculated bean plants.

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GROWTH OF SMALL FREE-LIVING AMOEBAE IN VARIOUS BACTERIAL AND IN BACTERIA-FREE CULTURES[1]

SHIH L. CHANG

Abstract

Hartmannella glebae, *H. agricola*, and *Naegleria gruberi* fed on a *Flavobacterium* sp. developed tolerance for the yellow pigment. Differences in the formation of plaques in the bacterial growth and in the growth cycle were observed.

Various species of bacteria were equally effective as food for all three amoebae, and richness of the amoeba cultures paralleled the richness of the growth of edible bacteria. Failure of *Pseudomonas pyocyanea* to support growth of these amoebae was attributed to the toxicity of the pyocyanin pigment, and inability of the amoebae to develop tolerance to this pigment was attributed to the relatively high solubility of the pigment in water.

These amoebae showed no growth on agar in the absence of living edible bacteria; however, bacteria-free *H. agricola* and *N. gruberi*, but not *H. glebae*, were successfully maintained in monkey kidney tissue cultures. The amoebae exhibited no toxic effect on the epithelial cells and fed on cell debris. Ingestion of very large numbers of cysts by young cells or any number by old cells resulted in degeneration of the cells with a release of live cysts. Epithelial cell degeneration hastened by enteroviruses facilitated rapid growth of the amoebae.

Introduction

In a preceding report (1), it was shown that luxuriant growth of the flagellate amoeba, *Naegleria gruberi*, in "pure mixed" cultures can be maintained indefinitely if the agar medium supports the growth of the bacterial associate on which the amoeba feeds, and if the medium itself is not inhibitory to the growth of the amoeba.

To determine whether the new culture method could be applied to other species of small free-living amoebae, and whether different species differ in their cultural characteristics, two strains each of *Hartmannella glebae* and *H. agricola* were grown by the same technique applied to *N. gruberi*. The growth characteristics of the two *Hartmannella* species and *N. gruberi* on a *Flavobacterium* sp. were studied in the same manner as was growth of the latter amoeba on *Proteus mirabilis*.

Growth of all three species of amoebae on a number of species of bacteria was ascertained quantitatively in terms of population densities and growth rates. Attempts were also made to grow the two *Hartmannella* species in the absence of bacteria, and all three species in monkey kidney tissue cultures. The results of these investigations form the basis of this report.

Materials and Methods

1. Amoebae

The history of the strain of *N. gruberi* has already been described (1). The two strains each of the *Hartmannella* species were recovered from river and well waters on a buffered sucrose nitrate agar containing 100 units/ml of

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Contribution from the Robert A. Taft Sanitary Engineering Center, U.S. Department of Health, Education, and Welfare, Cincinnati, Ohio.

penicillin and 20 μg/ml of streptomycin (1), and were isolated from their natural bacterial associates by the giant colony technique of Oehler (4). Since *Flavobacterium* sp. was found to support a rich growth of *N. gruberi* after a brief period of weak antiamoebiosis, and could be easily replaced by other edible bacteria, this bacterium was employed as the initial associate in these *Hartmannella* cultures.

H. glebae is a relatively large species among the small amoebae. The average diameter of rounded trophozoites is about 25 μ. The cysts have a single wall which is not "pierced" by pores. *H. agricola* is a rather small species. The rounded trophozoites average 12 μ in diameter and the cystic wall is similar to that of *H. glebae.* The identification of these two amoebae was based on characteristics of the mitotic changes described by Singh (6). The genus *Hartmannella* in Singh's classification (6) of small amoebae is synonymous with the genus *Acanthamoeba* (7).

2. Bacteria

Except *Bacillus globigii*, which was obtained from the American Type Culture Collection, all the other species of bacteria used in the study have been described in the preceding report (1).

3. Cultivation

The agar media, the method of making transfers, and the direct examination of plate cultures have also been previously described (1). In studying the feeding of amoebae on bacteria and in examining cultures of amoebae on *Aerobacter aerogenes*, whose mucoid growth hampered recognition of the trophozoites, a phase contrast microscope was used.

The monkey kidney tissue cultures were the same as used in a separate study, and their preparation has been described in another report (2).

Results

1. Temporary Antibiotic Effect of the Yellow Pigment of the Flavobacterium sp. on both H. glebae and H. agricola

As was observed with *N. gruberi*, both *H. glebae* and *H. agricola* exhibited poor growth in the first few subcultures of their association with the *Flavobacterium.* Death of those trophozoites that penetrated deeply into the bacterial growth was again noticed. It was not until after four or five subcultures that the amoebae were found to show increasing growth with each transfer. By the seventh or eighth subculture, growth of the amoebae apparently reached the richness normally attained by the present method of cultivation.

A crude acetone extract of the yellow pigment from the *Flavobacterium* cultures was found to be slightly soluble in water. A saturated aqueous solution of this pigment inhibited feeding on edible bacteria and within 48 hours caused death of trophozoites harvested from the early cultures. A half-saturated solution still showed the inhibitory effect on feeding, but caused no death of trophozoites within 48 hours. These effects were not observed on trophozoites harvested from later cultures which had developed tolerance.

The dried pigment extract was insoluble in water and exerted no toxic effect on trophozoites from early or later cultures, a phenomenon similar to that observed by Singh (5) on the dried crude pyocyanin extract.

These results, together with those observed earlier on *N. gruberi*, clearly indicate a toxic effect of the yellow pigment on free-living amoebae. Acquisition of a tolerance for this pigment, however, can be attributed to its extremely low solubility in water, which prevented diffusion into the agar to a toxic level and permitted the development of tolerance for very low pigment concentrations.

2. Growth Characteristics of the Amoebae on Flavobacterium

(a) Appearance

In 1 or 2 days of incubation at 25°–27°, or in about 1 day at 37°, with transfers made from active cultures, the growth of all three amoebae cultures resembled entirely that of the bacterial associate alone. This period of predominant bacterial growth would have been longer if transfers had been made from old cultures containing mostly cysts.

In cultures of *H. glebae*, small bacteriophage-like plaques were first visible 12 to 24 hours after the initial bacterial growth period. In another half-day or so, these plaques became enlarged and irregular in outline, and some of them coalesced to form larger plaques of irregular outline (Fig. 1). After 1 or 2 more days, the bacterial growth was so nearly superseded that only thin scattered patches were visible on close examination against light.

The appearance of plaques in cultures of *N. gruberi* was invariably 12–16 hours later than in those of *H. glebae*. In the early stage, they were more nearly circular and clearer than those formed by *H. glebae* (Fig. 2). Continuous enlargement of these small plaques resulted in the formation of one or two large, irregular plaques in the midst of many small ones (Fig. 2). In the large plaques there was always a limited return of bacterial growth which could be mistaken for an incomplete consumption of the original bacterial growth if the plates were not frequently examined. By the time the cultures became inactive, an irregular band of the initial bacterial growth remained around patches of the secondary bacterial growth.

On the other hand, no signs of growth of the *H. agricola* were visible during the first 8 or 9 days at 25°–27°, or 5 to 6 days at 37°. During the following week at the lower temperature, or 4 to 5 days at the higher, a light area developed in the center of the plate, became progressively lighter and larger, and formed a large plaque. Further expansion of the plaque during the next few days reduced the bacterial growth to an irregular band in the periphery (Fig. 3). The plate then became so dry that growth of the amoeba was slowed down and the remaining bacterial growth was consumed in 7 to 10 days. For the same reason of drying, secondary bacterial growth in the plaque was not seen.

In moisture-saturated incubators in which the plates showed very little if any drying even after weeks of storage, the cultures of *H. glebae* and *N. gruberi* were similar in appearance to those incubated without moisture

control. The cultures of *H. agricola*, on the other hand, showed a considerable delay in plaque formation. In plates on which there was an unbroken sheet of bacterial growth, the first appearance of the plaque could be delayed as long as 23 days, and it usually appeared in the periphery instead of the center. The log growth phase was shortened by 1 or 2 days.

(b) Growth Curve

Like that of *N. gruberi* on *Proteus mirabilis*, the growth curve of each of these three amoebae on the *Flavobacterium* consists of three distinct phases: lag growth, log growth, and retardant growth – encystment.

The duration of each phase was ascertained for each amoeba, and the corresponding population densities were estimated. From the population increase during the log growth, the values of the growth rate k and the generation time g were computed. Since no significant differences were observed between strains, distinctions were made only among species. The results of these determinations are shown in Table I.

TABLE I

Various values in growth curves of three species of free-living amoebae on *Flavobacterium* organism

Growth phase	Temp., °C	*H. glebae*	*H. agricola*	*N. gruberi*
Lag growth phase (hr)	25–27	6–8	150–160	4–6
	37	3–4	72–84	2–3
Log growth phase (hr)	25–27	60–66	84–96	56–60
	37	48–56	56–60	36–40
Retardant growth – encystment phase (days)	25–27	30–35	30–35	6–7
	37	20–30	20–30	4–5
Approximate number of amoebae per plate at growth peak	25–27	1×10^6	10×10^6	10×10^6
	37	6×10^5	15×10^6	15×10^6
Value of k/hr during log growth	25–27	0.57	0.72	0.66
	37	1.06	1.38	1.30
Value of g (hr) during log growth	25–27	5.25	4.16	4.55
	37	2.68	2.20	2.40

Several points of interest are brought out when the data in Table I are analyzed in the light of direct microscopic findings. First, the lag growth phase of *H. agricola* was at least 20 times as long as that of *H. glebae* or about 30 times that of *N. gruberi*. Direct microscopic examinations revealed that, soon after the transfers were made, the trophozoites of *H. agricola* were moving around among the smeared bacterial cells. When the latter started to form visible colonies, the former showed a tendency to move away from bacterial growth. Those that were buried in the bacterial growth either formed cysts or perished. The trophozoites that were outside the bacterial growth apparently managed to feed on bacterial cells in the periphery of minute colonies. Some of them formed cysts while others wandered about on open areas and showed very little growth activity until the log growth phase began.

The failure of *H. agricola* growth in the continuous sheet of bacterial growth on plates incubated under saturated moisture was attributed to death of trophozoites and cysts caused by the active bacterial growth. This,

PLATE I

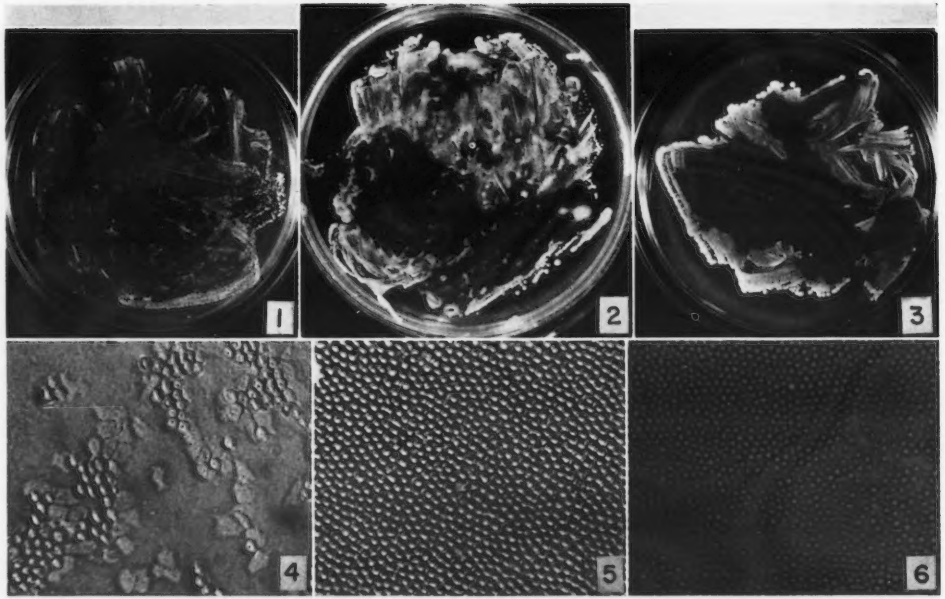

FIG. 1. Three-day-old culture of *H. glebae* on *Flavobacterium* sp. at 25°–27°.
FIG. 2. Five-day-old culture of *N. gruberi* on *Flavobacterium* sp. at 25°–27°.
FIG. 3. Eighteen-day-old culture of *H. agricola* on *Flavobacterium* sp. at 25°–27°.
FIG. 4. Patches of cysts and trophozoites of *H. glebae* in the interior of a large plaque. Magnification, ×100
FIG. 5. A sheet of cysts of *N. gruberi* in the interior of a large plaque. Magnification, ×100
FIG. 6. A sheet of cysts of *H. agricola* in the interior of a large plaque. Magnification, ×100

together with the fact that the log growth began at a time when the yellow pigment was intense, suggests that metabolic products other than the pigment accumulated during the active bacterial growth are detrimental to *H. agricola*.

Second, the log growth phase of *H. agricola* was considerably longer than that of either *H. glebae* or *N. gruberi*. This difference may be explained by the ability of the former amoeba to carry on growth activity without encystation in a relatively dry environment, which induced encystment of the latter amoebae.

Third, the retardant growth – encystment phase of the *N. gruberi* was about one-fifth that of either species of *Hartmannella*. This much-shortened last phase of *N. gruberi* was apparently a result of its early completion of encystment.

Fourth, encystation of all three amoebae was found to occur in the interior of large plaques simultaneously with multiplication of the trophozoites in the periphery. This, however, was not observed previously with *N. gruberi* grown on *P. mirabilis*. It appears that the stationary and relatively dry growth of *Flavobacterium* facilitated localization of amoebae colonies, whereas the swarming of the *Proteus* made the cultural environment more or less uniform throughout the plate.

Fifth, excystation of *N. gruberi* during the retardant growth – encystment phase was very rare. It was also infrequent for *H. agricola* but frequent for *H. glebae*. Thus, in the interior of plaques formed by *N. gruberi* and *H. agricola*, a monolayer of cysts was found (Figs. 5 and 6), and in that formed by *H. glebae*, isolated patches of cysts and trophozoites (Fig. 4). It appears that a return of bacterial growth stimulated excystation of *H. glebae* but not of *N. gruberi*.

Sixth, with the growth of a significant number of trophozoites extended into the retardant growth – encystment phase, the population of *N. gruberi* reached a much higher level on *Flavobacterium* than on *P. mirabilis*, and approached that of *H. agricola*. The population of *H. glebae*, on the other hand, was only 1/10 as great at 25° to 27°, and even smaller at 37°. In this connection, it is interesting to note that *H. glebae* was a decidedly more active consumer of bacteria than either of the other two amoebae. Because of its rapid bacterial consumption, especially at 37° C, the trophozoites in the retardant growth – encystment phase frequently became reduced in number without an increase in cysts, suggesting that some of them died of starvation before they were able to encyst.

Seventh, the values of k or g obtained for these three amoebae suggest that the larger the amoeba, the slower is its growth rate. The difference in values of these growth constants obtained at two different temperatures indicates again that a 10-degree rise in temperature within the physiological range nearly doubled the growth rates of all three amoebae.

3. Replacement of the Flavobacterium sp. in Pure Mixed Cultures of the Amoebae by Different Bacteria

Using the giant colony technique, replacement of the *Flavobacterium* associate in cultures of the two *Hartmannella* species by various species of

bacteria was carried out in the same manner as described in the preceding report (1). The ability of the amoebae to feed and grow on these bacteria was again determined separately. The results of these experiments are shown in Table II, together with those obtained previously on *N. gruberi*.

TABLE II

Ability of three species of free-living amoebae to feed and grow on various species of bacteria

Replacing bacterium	Edibility by:			Growth in culture of:†		
	*H. g.**	*H. a.**	*N. g.**	*H. g.*	*H. a.*	*N. g.*
Salmonella typhosa	Edible	Edible	Edible	++	++	++
S. schottmuelleri	Edible	Edible	Edible	++	++	++
S. paratyphi	Edible	Edible	Edible	++	++	++
S. typhi-murium	Edible	Edible	Edible	++	++	++
S. cholerae-suis	Edible	Edible	Edible	++	++	++
S. meleagridis	Edible	Edible	Edible	++	++	++
S. enteritidis	Edible	Edible	Edible	++	++	++
S. pullorum	Edible	Edible	Edible	+	+	+
Shigella dysenteriae II	Edible	Edible	Edible	+	+	+
S. sonnei	Edible	Edible	Edible	++	++	++
Escherichia coli	Edible	Edible	Edible	+++	+++	+++
A. aerogenes (*Klebsiella*)	Edible	Edible	Edible	++++	++++	++++
Bacillus subtilis	Inedib.	Inedib.	Inedib.	None	None	None
B. globigii	Inedib.	Inedib.	Inedib.	None	None	None
Pseudomonas pyocyanea	?	?	?	None	None	None

*H. g., H. glebae; H. a., H. agricola; N. g., N. gruberi.
†Richness compared with that on *Flavobacterium* sp.: ++++ comparable; +++ about 75%; ++ about 50%; + about 25%.

As to be expected, there was a parallelism between the edibility of the bacteria and their ability to support growth of amoebae. There was also a parallelism between the richness of the growth of all three amoebae and that of the bacterial growth of the edibile species. These observations show that the amount of amoeba growth depends primarily on the supply of edible cells.

The following cultural findings deserve mentioning. First, the growth of all three amoebae on *A. aerogenes*, which produced a mucoid growth not unlike that of *P. mirabilis*, was very similar to their growth on the latter bacterium, indicating that the nature of the bacterial growth is a controlling factor determining the growth characteristics of these amoebae. Second, the lag growth phase of all three amoebae, particularly *H. glebae* and *N. gruberi*, was considerably prolonged in their association with *E. coli*. Third, the lag growth phase of *H. agricola* on the *Salmonella* and *Shigella* species was shortened to about one-half of that on *Flavobacterium*. Since growth of these enteric pathogens was relatively slow under these cultural conditions, it is believed that the earlier start of the amoeba log growth was a result of low accumulation of the metabolic products suppressive to the growth of the amoeba.

Third, the thoroughly washed cells of *Pseudomonas pyocyanea* were edible by all three amoebae, while unwashed cells were not. Furthermore, the trophozoites dispersed in the suspension of the unwashed cells were found dead after 24 hours. These observations were in agreement with that made previously on *N. gruberi* alone (1). Additional observations made with a crude extract

of pyocyanin produced results indicating a toxic effect of the pigment to all three amoebae very similar to that observed by Singh (5). It seemed then that the non-supportability of *P. pyocyanea* for the growth of these amoebae was due not to the inedibility of its cells but to the toxic effect of its pigment.

In an effort to find out whether these amoebae could develop tolerance for the pyocyanin as they did for the yellow pigment, an experiment was made in which a subtoxic concentration (1:10,000) of the crude extract was incorporated in the agar medium used in growing these amoebae on the *Flavobacterium* organism. After every three subcultures, the concentration of the extract was increased by 20%. Positive cultures of all three amoebae were obtained until the extract concentration reached a 1:2000 level, at which negative cultures were uniformly observed. Although these amoebae had thus acquired a partial tolerance for the pigment, attempts to grow them in association with the *P. pyocyanea* were still failures.

In agreement with the observation of Singh (5), the dried crude pyocyanin extract was insoluble in water and nontoxic to the amoebae.

4. Growth of the Amoebae in Absence of Living Bacteria

By the giant colony technique and subsequent transfer of the migrated trophozoites onto fresh agar plates, bacteria-free amoebae of either species of *Hartmannella* were isolated. Like those of *N. gruberi* prepared previously, these trophozoites rapidly underwent encystation, and the cysts failed to excyst unless living edible bacteria were added. In the case of *H. glebae*, the number of cysts formed were always a little smaller than the number of trophozoites planted, indicating that some trophozoites died of starvation before they were able to encyst. Enrichment of the agar with nicotinic acid, pantothenic acid, pyridoxine, riboflavin, para-aminobenzoic acid, thiamin, inositol, ascorbic acid, biotin, and cholesterol failed to alter the ill fate of the amoebae. Heat-killed edible bacteria again gave a very limited amount of amoeba growth for a short period of time.

5. Growth of the Amoebae in Monkey Kidney Tissue Cultures

Since free-living amoebae were reported to have contaminated monkey kidney tissue cultures from a commercial source (3), attempts were made to grow these amoebae in monkey renal epithelial cell tissue cultures. It is interesting that successes were met with in the first attempt with both *N. gruberi* and *H. agricola*, and failures with *H. glebae*. The richness of these cultures was, however, less than that attained by Jahnes *et al.* (3).

In a series of some 60 subcultures, the following outstanding features have been observed. No toxic effect of the amoebae on the epithelial cells was noticed. The trophozoites moved about freely over the epithelial cells, but fed entirely on cell debris; therefore, their growth was governed by the number of cells undergoing degeneration. Cysts were readily phagocytized. When cysts were ingested in not too large numbers by cells of young cultural age, they were usually digested; but when they were ingested in very large numbers by young cells, or in any number by old cells, degeneration of these cells was

hastened. Disintegration of the cells released live cysts which soon excysted. The amoeba growth became rapidly and progressively richer as the cycle of cyst ingestion and release was repeated at the advanced cultural age.

It is of particular interest that when the CME-456 strain of Coxsackie A9 virus, the Wallace strain of Echo 7 virus, or the Mahoney strain of poliovirus type 1 was added to these tissue cultures, in which they produced a complete lysis of the epithelial cells in 3 to 6 days, the amoebae grew into rich cultures in 6 to 9 days and became encysted when the cell debris was consumed. No evidence was observed of infection of the amoebae by these viruses.

These tissue-culture-grown amoebae could also be maintained on agar plates if they were supplied with the epithelial cells or cell debris in each transfer. Cells or cell debris heated at 60° C for 30 minutes supported very little if any amoeba growth, indicating again the thermolabile nature of the growth factor.

Discussion

While problems concerning nutrition of the free-living amoebae are not solved by the present experimental approach, this type of study nevertheless facilitates observation of their cultural characteristics in a reproducible manner and reveals the complexity of their nutrition.

As brought out in the present study, these amoebae are similar in their general nutritional requirements and susceptibility to toxic effects of bacterial pigments, but differ in many of their growth characteristics. Accumulation of information related to the latter will aid in identification and classification of the small amoebae.

The relatively slight differences in growth rates of these three amoebae substantiate the previous statement that amoebae as a whole multiply under favorable conditions much faster than either ciliates or haemoflagellates (1). The insignificant difference in growth rates of *N. gruberi* on *Flavobacterium* and on *P. mirabilis* indicates that the growth rate of this amoeba is not influenced by association with these different species of bacteria.

While the number of species of bacteria employed in the study was too small for generalization, the results suggest that Gram-negative bacilli are edible by the amoebae and that Gram-positive bacilli are not. Should this prove to be true, it would be very interesting to find out why.

The success of the amoebae in developing tolerance for the yellow pigment of *Flavobacterium* and their failure to do so for the pyocyanin extract at concentrations of 1:2000 and higher present an interesting phenomenon. It should be added that in the period during which the tolerance was being developed, trophozoites of the *N. gruberi* and the *H. glebae* were found to move away from the bacterial growth in the first 24 to 36 hours, and significant numbers of them were not seen in the latter until 3 or 4 days of incubation had elapsed. This delayed invasion of the bacterial growth was much more pronounced with the *H. agricola*. Since the yellow pigment was most intense on the fourth and fifth days of incubation, it appeared that the cytotoxic

substance was probably not the insoluble yellow pigment but something else which could be either the predecessor of the yellowing pigment or a toxin liberated along with it. Singh (5) suggested the same explanation for his observation on the toxic effect of the water-insoluble pigment of *Chromobacterium prodigiosum* on two unidentified soil amoebae.

The failure of *H. glebae* to grow in the monkey kidney tissue cultures, against the success of the other two amoebae, remains to be explained.

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STUDIES ON THE METABOLISM OF LEAVES WITH LOCALIZED VIRUS INFECTIONS

I. OXYGEN UPTAKE[1]

M. WEINTRAUB,[2] W. G. KEMP, AND H. W. J. RAGETLI[2]

Abstract

Infection of *Nicotiana glutinosa* with tobacco mosaic virus, of *Dianthus barbatus* with a carnation virus, of *Gomphrena globosa* with potato virus X, and of *N. tabacum* with two strains of turnip mosaic virus produced local lesions and significantly increased O_2 uptake to an extent dependent upon the stage of infection. Increased respiration in *N. glutinosa*, as measured by O_2 uptake, was detected 7–10 hours before lesions appeared. When infected plants were maintained at 32° C, the rise in respiration occurred 8–15 hours before the appearance of lesions, indicating an increase or prolongation of virus multiplication. Local lesion infection of other hosts, though differing in detail, had essentially the same effect. Non-virus injury, including artificially produced necrotic local lesions, did not induce a similar increase in respiration. When necrotic areas were excised from infected disks the remaining portions still respired at a greater rate than healthy controls. Increased respiration appeared to be correlated with virus activity other than the necrotic process.

Introduction

In a study on the inhibiting effect of certain heterocyclic compounds upon virus infection (7), it was felt desirable to learn something of the possible physiological effects of these compounds. At that time, there was nothing in the literature about the respiration, and little about the general metabolic effects, of localized virus infections upon the host. All such studies had been done with systemically infected plants (e.g. refs. 6, 9, 10). It became necessary, therefore, to study such localized infection systems, and a portion of the results has already been reported (7). Since then, Owen (4) and Yamaguchi and Hirai (11) have confirmed our reported observation that respiration rises markedly in leaves with localized necrotic virus infections. The present paper reports further details and subsequent experiments.

Materials and Methods

Tobacco mosaic virus (TMV) inoculations were made with purified virus, while inoculations with potato virus X (PVX), the carnation virus (CMV), and the turnip mosaic viruses (TpMV) were made with sap from homogenized leaves. The CMV was originally isolated from greenhouse carnations, and has been tentatively identified as being similar to the carnation mottle virus of Kassanis (3). The two strains of TpMV, Nova Scotia and Quebec, have been described (1) and were obtained from infected turnip plants.

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Contribution from Plant Pathology Laboratory, St. Catharines, Ontario.
[2]Present address: Plant Virus Unit, Research Branch, Canada Department of Agriculture, c/o The University of British Columbia, Vancouver, B.C.

All inoculations in the work reported here were done with the aid of carborundum dusted on the leaves, and were followed by thorough rinsing of the inoculated leaves with water. Since the desired result of inoculation was local lesions *not* followed by systemic infection, *Nicotiana glutinosa* was used with TMV, *Dianthus barbatus* with CMV (2), *Gomphrena globosa* with PVX, and *N. tabacum* with TpMV.

The time of appearance of TMV lesions in *N. glutinosa*, which varied considerably with the age of the plants, the season of the year, and the conditions under which the plants were kept before and after inoculation, ranged from as little as 24 hours to (occasionally) more than 50 hours. Such variation was somewhat reduced by placing the plants in growth chambers for several days before inoculation, and until the plants were used for the experiment after inoculation. The chambers were constant for temperature and light, the former at about 22° C, the latter at 1200 ft-c for 18 hours a day.

Respiration was measured by determining the oxygen uptake of leaf disks, 11 mm in diameter, cut out with a cork borer from leaves inoculated with virus. Conventional Warburg manometry was used, except that to accommodate four disks each, the vessels lacked a center well, and KOH was therefore placed in one of the side-arms.

Since many experiments were carried out before lesions developed, a concentrated inoculum was used, except where otherwise noted, to ensure infection of disks cut from inoculated leaves. The remaining portions of the inoculated plants were kept in the dark, to make certain that lesions did develop, and to determine the time of their appearance.

Disks cut from uninoculated leaves were used as controls. In most experiments these disks were taken from another plant carefully selected to match the inoculated plant in size and general appearance. In some experiments only a half-leaf was inoculated, the other half serving as a source of control disks. There was, however, no difficulty in matching experimental and control plants, especially since leaves in the same relative stem-position were used. Differences in respiratory activity among replications of healthy controls in any one experiment were found to be statistically insignificant.

In the early experiments leaves from which the healthy control disks were cut were also rubbed with carborundum and water. However, this procedure was discontinued when it was found that under the conditions reported here the respiration of rubbed leaves did not differ from that of unrubbed leaves (8).

The actual measurements of oxygen uptake were made in the dark at 25° C, the Warburg vessels containing either 2 ml of distilled water if the experiments were of short duration, or 2 ml of Vickery's solution if of longer duration. All experiments were run in duplicate or quadruplicate. A period of 20–30 minutes was allowed for equilibration before initial readings were taken and this equilibration period should be added to any time figures. Results were calculated as μl O_2 per hour per 100 mg fresh weight (F.W.) or as μl O_2 per hour per 10 mg dry weight (D.W.). Student's '*t*' test for significance was applied to the results of all experiments, and an analysis of variance to some. Whenever significance is noted in the text, this refers to $P = .001–.01$.

Results

Respiration of TMV-infected N. glutinosa

Preliminary experiments showed very quickly that in leaves on which the necrotic lesions were well developed, the respiration was considerably and significantly greater than that of healthy controls. A typical series (Table I) shows the gradual rise in respiration of the infected leaf disks as the course of infection proceeded. In these experiments series of plants were inoculated at 6-hour intervals, starting 48 hours before the respiration run, at the beginning of which disks were cut from each pair of healthy and diseased plants. Lesions were present on disks inoculated for 24 hours or longer. There was considerable variation in the respiration of plants in the different time periods as shown in Table I (e.g. healthy at 12 hours, and at 42 hours), because the experiments, involving as they did so many replications, had of necessity to be done at different times and on different plants. There was, however, no significant difference within each time period between duplicates of healthy disks or between duplicates of diseased disks. This reproducibility was due to the careful matching of healthy and diseased leaves for each time period, and so any differences in respiration would be due to the infection.

These experiments show that a significant rise in respiration occurred at 24 hours, at which time lesions had appeared. This rise reached a maximum at 30 hours, after which respiration began to decrease, as calculated either on a F.W. or D.W. basis. However, by 42 hours there was an apparent divergence in the ratio of diseased to healthy respiration (D/H) depending upon which weight basis was used (see Table I). The reason for this was that the infected leaves were becoming greatly desiccated at this later stage of infection, so that the F.W. of infected disks was one quarter or less that of healthy disks. Since calculations made on a 100-mg F.W. basis did not take this loss of water through necrosis into account, a mathematical overcompensation resulted, giving rise to an illusory increase in respiration. It was found in large numbers of experiments that in early stages of infection results calculated on a F.W.

TABLE I

Respiration (μl O_2/hour per 100 mg F.W. and per 10 mg D.W.) of leaf disks from *N. glutinosa*, inoculated with TMV (D), and healthy (H)

Hours after inoculation	D		H		Ratio D/H	
	F.W.	D.W.	F.W.	D.W.	F.W.	D.W.
0	30.7	37.0	31.2	38.0	0.98	0.97
6	25.7	29.4	29.1	29.3	0.89	1.00
12	38.0	38.4	35.9	37.7	1.06	1.02
18	37.0	40.8	34.7	41.5	1.04	0.98
*24**	*33.4*	*35.8*	*22.7*	*28.3*	*1.47*	*1.27*
30	48.4	49.2	26.5	32.2	1.83	1.53
36	41.3	30.0	34.3	39.7	1.20	0.75
42	32.5	13.3	19.2	23.7	1.70	0.56
48	41.1	15.2	26.7	32.5	1.54	0.47

*Italicized data were from the first set of disks in the time series showing the presence of lesions.

basis were not significantly different from those calculated on a D.W. basis, whereas at the later stages of infection only the D.W. basis was valid.

Subsequent experiments confirmed the observation that whenever lesions appeared, a rise in respiration occurred. However, since a considerable variation was found from experiment to experiment in the time of initial appearance of lesions, it was obvious that the best way to find a correlation between the rise in respiration and appearance of lesions was to follow the oxygen uptake of disks continuously from the time of inoculation until lesions were formed both on the remaining leaf portions on the plant and on the disks themselves.

Accordingly, disks were cut from inoculated plants immediately after inoculation, and from healthy plants that had been rubbed with water and carborundum. The disks were floated on Vickery's solution in the Warburg vessels, and the source plants were placed in a dark chamber and examined every half-hour for the first indications of symptoms. Respiration of the excised disks was then followed continuously for 51 hours, with readings every 1/2 hour. Figure 1 shows the respiration of two of eight replications. All the replicates but one followed very closely the pattern of the two shown.

The first appearance of symptoms in this experiment occurred 37 hours after inoculation (as marked in Fig. 1). All the seven sets of infected disks were respiring at a significantly increased rate over the healthy controls from 7 to 10 hours before lesions appeared on the remaining portions of the inoculated plants. It is quite probable that this is a conservative estimate of the time, since experience has shown that detached leaves or portions of leaves

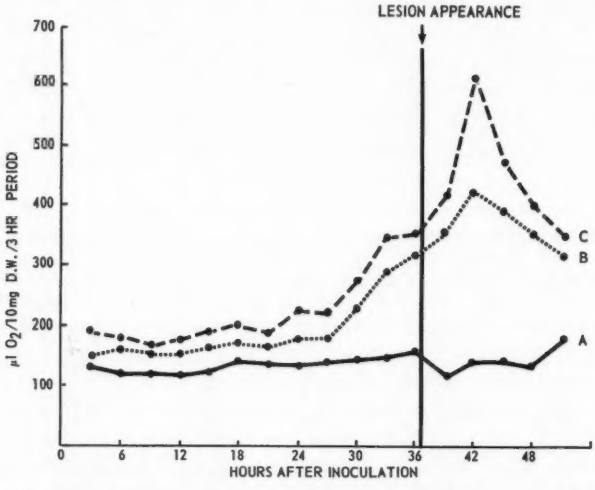

FIG. 1. Respiration as measured by O_2 uptake of disks of healthy and TMV-infected *N. glutinosa* leaves, measured continuously for 51 hours. •———• A: healthy disks; •— — — —• B, and •—— ——• C: diseased disks. Vertical line marked with arrow indicates the first appearance of lesions, 37 hours after inoculation.

floating on water or other solutions require a somewhat longer time to develop symptoms than do leaves attached to the plant (7). Since, in this experiment, lesions were considered to have occurred when the first pock-like depressions were observed on the leaves, it is evident that a significant rise in respiration took place before any overt morphological changes in the leaf.

In the single deviating replicate, oxygen uptake increased continuously from the 45th to 48th hour until the end of the experiment. When the disks were removed from the vessels for D.W. determination, it was found that unlike the other seven replicates, which had produced lesions, the deviating set had no virus lesions at all. Furthermore, when a bacterial count of the Vickery's solution in each vessel was made, it was found that neither the healthy nor the seven lesioned replicates contained enough bacteria to contribute significantly to the observed rise in respiration, but that the solution that had contained the deviating replicates did have an abnormally high bacterial count, sufficient to account for the sudden increase in O_2 uptake at 45 hours.

Respiration of Leaves with Virus Multiplying at High Temperatures

The previous experiments indicated that the rise in respiration was correlated with virus activity other than necrotic processes per se, since a significant increase preceded necrotization, although undoubtedly increasing necrosis did accompany the maximum increase in respiration. If the activity responsible for this increase was virus multiplication, then any method that might enhance or prolong this activity, without at the same time doing away with the localization of the virus, should result in a proportionately longer period of increased respiration before appearance of symptoms.

Samuel (5) showed that between 28° and 35° C TMV produces lesions on *N. glutinosa* that are less localized than at lower temperatures, and tend to coalesce. Tests in a constant temperature chamber showed that under our conditions 32° C was an ideal compromise temperature. Here there was a tendency for the virus to become systemic, as evidenced by the blotchiness and chlorotic quality of the lesions, while at the same time lesions could be readily detected. The respiration of disks from plants inoculated with TMV and held at 32° C was measured. Within 36 hours of inoculation, the respiration of these disks was 65.9% higher than that of the healthy controls. At this time, there was no evidence of symptoms on the leaves left in the darkened constant temperature chamber, nor, at the end of the experiment, on the disks themselves. Forty-four hours after inoculation the leaves in the chamber had two or three small chlorotic areas, which might possibly have been incipient lesions, but it was not until 51 hours after inoculation that symptoms could definitely be said to be present.

In this experiment, another set of controls that was taken from inoculated plants kept in the greenhouse at temperatures ranging from 20° to 24° C showed that the respiration of the infected disks was, at 36 hours, just beginning to increase significantly over that of the healthy controls from the greenhouse, although the increase was of a low order of magnitude (23.3%), at this early stage of lesion development.

Effect of Non-virus Lesions on Respiration

Although the evidence indicated that increased respiration was not the result of necrotic processes, the effect of artificially produced necrotic lesions of non-virus origin was examined. Such lesions were produced by spraying leaves of *N. glutinosa* with a 1% $CuSO_4$ solution. Forty-eight hours after spraying, small brown necrotic lesions, closely resembling those of TMV in appearance, were present. Since there was a possibility that the copper itself might inhibit respiration, an additional control was included in disks cut from leaves sprayed with 1% $CuSO_4$ immediately preceding the experiment. Two other sets of disks were included, one from leaves inoculated with TMV on which virus lesions were well developed, and one from healthy untreated leaves. Only the disks taken from the TMV-infected leaves respired at a significantly higher rate than any of the others. The treatment with 1% $CuSO_4$ did not, therefore, result in increased respiration, although localized necrosis was present in the leaf disks.

To determine whether a generalized injury might result in a respiratory increase, *N. glutinosa* leaves were immersed in water at 45° C for 5 minutes. Control disks were cut from leaves dipped in water at room temperature. The respiration of these disks was compared with that of the disks from TMV-inoculated leaves on which lesions were well developed and the disks from healthy untreated leaves. Only the TMV-infected disks respired at a significantly higher rate than any of the others. The set of leaves dipped in water at 45° C respired at a lower rate than the others, although the difference from the control was of a low order of significance ($P = .05$). There was no significant difference between the respiration of the healthy leaf disks and the controls dipped in water at room temperature. It was concluded that a generalized injury, rather more severe than localized necrosis, resulted in a decrease in respiration, whereas a non-virus localized injury such as produced by 1% $CuSO_4$ did not result in an increase in respiration.

Excision of Virus Lesions

The question now arose as to how much the necrotic areas in virus lesions were actually contributing to the rise in respiration. To determine this, experiments were run on disks from which the virus lesions were punched. The lesions were allowed to develop on the TMV-inoculated *N. glutinosa* leaves until it was expected, from experience, that respiration would be close to its maximum. At this time, the disks were cut from the infected leaves, and each of the necrotic lesions was punched out with a specially made borer which cut a hole of 0.79-mm diameter. This was found sufficient to remove all the necrotic tissue. Control disks from healthy leaves were treated in the same way, each disk being matched with an infected disk, and the small holes being punched out in the same number and pattern as the infected ones. Further controls were run with intact disks from both infected and healthy leaves.

Sufficient time (3–4 hours) was given the disks to recover from the initial injury caused by the perforation before O_2 uptake determinations were begun.

In these experiments (Table II) no significant difference was found between the intact healthy and perforated healthy disks, nor was there any significant difference between the intact infected and perforated infected disks, but there was a significant difference between the infected and healthy disks, the respiration of infected disks being almost double that of the healthy ones.

TABLE II

O_2 uptake of TMV-infected and healthy *N. glutinosa* leaf disks, intact and perforated,* at time of maximum respiration in diseased disks

Condition of disks	Treatment of disks	μl O_2/hour per 10 mg D.W.
Healthy	Intact	21.9
	Excised	24.3
TMV-infected	Intact	44.5
	Excised	44.6

*"Perforated" refers to 11-mm diameter disks cut from the leaf, after which lesions were punched out from infected disks, and a similar number of excisions made from healthy disks, with a 0.79 mm borer. Disks of 11-mm diameter without further excisions are "intact".

Effect of Other Localized Virus Infections on Respiration

To see how general were the effects on respiration of local virus infections other than TMV on *N. glutinosa*, four other virus–host systems which produced local lesions were examined. As mentioned earlier, these were CMV on *D. barbatus*, PVX on *G. globosa*, and two strains of TpMV on *N. tabacum*. As might be expected, although the general respiration pattern was similar to that of TMV on *N. glutinosa*, the rate at which respiration reached a maximum varied, since the lesions in these other hosts developed quite differently and at different rates.

PVX on *G. globosa* ran its course of respiration in much the same way as TMV on *N. glutinosa*, except that the time scale was considerably extended. Three days after inoculation, the diseased tissues respired at a significantly greater rate than the healthy controls, and it was not until 8 days after inoculation that the respiration of the diseased disks began to fall and approach once again that of the healthy controls.

CMV on *D. barbatus* behaved somewhat differently. After 8 days the respiration of the infected tissues was still significantly higher than that of the healthy controls. This difference may have been due to the fact that the CMV lesions continued to enlarge slowly over an observed period of 2 weeks, so that there must have been continuous virus multiplication and invasion of new tissues throughout this period.

Finally, the two strains of TpMV presented a respiration pattern somewhat difficult to interpret. As shown in Table III, both strains, when inoculated to old leaves, in which they produced large numbers of lesions, resulted in an increased respiration 4 days after inoculation. On the other hand, young leaves, on which the number of lesions (although produced at the same time as those on old leaves) rarely exceeded one per experimental disk, did not

TABLE III

Respiration of various leaves locally infected with various viruses (lesions present in all infected material)

Virus–host	Days after inoculation	Age of leaf	μl O_2/hour per 10 mg D.W. (av. of two replicates)	
			Diseased disks	Healthy disks
PVX – *G. globosa*	3		18.4 (S)*	6.7
	5		15.9 (S)	8.0
	8		9.8 (N.S.)**	6.7
CMV – *D. barbatus*	3		12.6 (S)	6.6
	8		11.3 (S)	8.0
TpMV (Nova Scotia strain) – *N. tabacum*	4	Old	32.3 (S)	8.4
	5	Young	24.8 (N.S.)[a]	21.3
	11	Old	29.0 (S)[b]	11.0
	11	Old	26.3 (S)[c]	11.0
	11	Young	21.0 (S)[d]	16.1
TpMV (Quebec strain) – *N. tabacum*	4	Old	26.6 (S)	8.4
	5	Young	20.8 (N.S.)[e]	21.3

*S indicates significant difference between infected and diseased disks, Student's '*t*' test, P = .01–.001.
**N.S. indicates non-significance, P = or >.05.
[a]8 lesions, [b]25 lesions, [c]8 lesions, [d]8 lesions, [e]13 lesions, on the eight experimental disks.

respire at an increased rate 5 days after inoculation. However, this failure was not entirely due to the small number of lesions produced, since 11 days after inoculation, both old and young leaves showed an increase in respiration, even with a small number of lesions on both. (The number of lesions on the old leaves was reduced by dilution of the virus inoculum and careful selection of material.) Thus the total infected area is not, in this case, the only factor involved in increased respiration, and the physiological condition of the tissues involved plays some part.

Conclusions

All the localized virus infections studied gave rise to a marked increase in respiration of the diseased tissues, suggesting an intense metabolic disturbance.* The fact that a rise in respiration occurred some 7–10 hours before the appearance of symptoms when tissues were kept at 22° C, and a similar increase 8–15 hours before the appearance of symptoms when tissues were kept at 32° C, indicates strongly that the metabolic disturbance is correlated with virus multiplication rather than with the necrotizing injury caused by the virus. This conclusion is substantiated by the fact that infected tissues from which the necrotic lesions had been excised still respired at an increased rate. The failure of artificial injuries, including necroses similar in appearance to TMV lesions, to stimulate a comparable metabolic disturbance reinforces the con-

*A quantitative estimate of the intensity of the disturbance will be dealt with in a forthcoming publication.

clusion that the necrotic processes per se were not responsible for the metabolic changes. Indeed, as necrosis became more severe, the respiration of such tissues declined, eventually to a rate well below that of healthy controls.

These results would seem to be at variance with those obtained by Yamaguchi and Hirai (11), who concluded that the rise in respiration was the result of necrosis rather than of virus multiplication. However, their results are somewhat difficult to assess, since they claim to have been working with an "ordinary strain (of TMV) found in Japan" (11), which was capable of passing from an inoculated half-leaf of *N. glutinosa* to the uninoculated half within 5 days at 25° C. This is contrary to any reported, and our own, experience with the ordinary strain of TMV found in North America, or England (5), which at such temperatures does not become systemic in *N. glutinosa*.

When one considers the experiments in which the necrotic areas were excised, the magnitude of the metabolic disturbance involved in the respiratory increase is seen to be very large. Such a metabolic disturbance should also be correlated in some way with shifts in utilization and concentrations of metabolites. The studies made to determine such shifts will be reported in later publications.

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CAROTENOID PIGMENTS OF HALOPHILIC BACTERIA[1]

R. M. BAXTER

Abstract

The carotenoids of two yellow and four red halophilic bacteria were studied by chromatography on alumina. One yellow organism contained predominantly one pigment, or a group of closely related pigments similar to neurosporene, while the other contained the neurosporene-like pigments and appreciable amounts of an unknown pigment with absorption maxima at about 420, 450, and 485 mμ.

The red organisms, three species of *Halobacterium* and one *Sarcina*, contained α- and β-bacterioruberin, and at least six minor pigments. All except two of these were very similar to carotenoids previously reported in *Rhodospirillum rubrum*. Methoxyl could not be detected in α-bacterioruberin, supporting the suggestion of others that it is demethylated spirilloxanthin. It is suggested that the pathway of carotenoid biosynthesis in red halophiles is very similar to that in *Rhodospirillum rubrum*, except that the pigments are not methylated.

The carotenoid content of *Halobacterium salinarium* was not influenced by addition of various precursors to the growth medium, or by altering the physical conditions of growth, but increased with the age of the culture.

Introduction

Klebahn (12) first showed that the red halophilic bacteria owe their color to the presence of fat-soluble pigments. Petter (17) found two carotenoids in *Bacterium halobium* which she named α- and β-bacterioruberin. α-Bacterioruberin was the more abundant, and had absorption maxima at 467, 490, and 522 mμ in methanol. β-Bacterioruberin had maxima at 453, 482, and 504 mμ. Both pigments were hypophasic (i.e. entered the more polar phase) on partition between petroleum ether and methanol (80–92%). Lederer (13) found only α-bacterioruberin in this organism. He pointed out that its absorption spectrum was identical with that of spirilloxanthin and suggested that it might be a demethylated derivative of this pigment. Spruit and Pijper (18) found three pigments in a species of *Halobacterium* isolated from South African solar salts, and suggested that more might be present. Two of their pigments seem to correspond to Petter's α- and β-bacterioruberin; the third had its main peak at about 450 mμ in hexane.

Spirilloxanthin was first isolated by van Niel and Smith (20) from *Rhodospirillum rubrum* and is identical with rhodoviolascin isolated by Karrer and Solmssen (11) from a mixed culture of *Rhodovibrio*. It contains 13 double bonds in conjugation and two methoxyl groups, and has the empirical formula $C_{42}H_{60}O_2$. The structure proposed by Karrer and Koenig (10) was criticized by van Niel (19) and a revised and probably correct structure has recently been proposed by Liaaen Jensen (14).

In the last few years a good deal has been learned about bacterial carotenoids and it seemed worthwhile, therefore, to reinvestigate the carotenoids of the

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red halophiles in the light of more recent information. The pigments of two yellow halophiles were also examined.

Materials and Methods

The organisms used are from the collection of Dr. N. E. Gibbons. *Halobacterium salinarium*, *H. cutirubrum*, and *Sarcina littoralis* were originally obtained from Lochhead (16) and *H. halobium* from the Delft collection. A pale-yellow coccus, H5B-2, is a strain of H5 of Venkataraman and Sreenivasan (21). A bright-yellow coccus, SE 20-4, was isolated by Gibbons from saline soil from Saskatchewan.

The organisms were grown at 36° with aeration in 10-liter amounts of our usual medium (1) containing 25% sodium chloride for the red halophiles and 10% for the yellow ones. After incubation for 7 days, the cells were harvested in the Sharples centrifuge.

The pigments of the red-pigmented rods were easily extracted because the cells lyse in media of low salt concentration. The cell paste obtained from the Sharples was stirred vigorously in a Virtis homogenizer with about its own weight of water; 3 volumes of acetone were then added and the mixture again stirred. The cell residue was removed by filtration or centrifugation and washed two or three times with acetone.

The red sarcina and the yellow cocci do not lyse in water and the extraction of their pigments is therefore less complete than from the halobacteria. The most effective method seemed to be shaking the cells in acetone with small glass beads (ballotini), although the residues never became completely colorless.

The solutions containing the pigments were left at room temperature for a few minutes after the addition of methanolic potassium hydroxide to saponify any esters that may have been present. Petroleum ether* was then added and sufficient 5 *M* sodium chloride solution to transfer the pigments to the petroleum ether layer. The aqueous phase was extracted once or twice with petroleum ether and discarded. The petroleum ether solution was then washed several times with sodium chloride solution and distilled water.

The washed petroleum ether solution of the pigments from the yellow organisms was dried over anhydrous sodium sulphate solution and the solvent was removed by evaporation under reduced pressure in a stream of nitrogen or nitrogen and carbon dioxide. However, when the petroleum ether solution of pigments from the red organisms was washed free of acetone and methanol, most of the pigment precipitated as a very dark red flocculent material at the interface. This was removed by filtration and redissolved in a mixture of petroleum ether and acetone. This solution and the petroleum ether solution containing the minor pigments were then treated in the same way as the extracts of the yellow organisms. The dried material was stored at −15° C until used.

*Throughout this paper, petroleum ether refers to the fraction of boiling range 30–60°.

Pigments were separated on aluminum oxide (Merck reagent, "suitable for chromatography") used without deactivation for the pigment of the yellow organisms but after partial deactivation with methanol (4) for the pigments of the red organisms. The columns, 2-cm inside diameter, were packed to a height of about 7 cm by pouring in a slurry of alumina in *n*-hexane. The hexane was then removed by washing with petroleum ether. This procedure was used because columns made with a slurry of alumina in petroleum ether almost always developed cracks and pockets.

The dried pigments were redissolved in petroleum ether, except the petroleum ether insoluble fraction from the red halophiles, which was dissolved in a mixture of petroleum ether and acetone (9:1), and placed on the columns. The chromatograms were developed with 100-ml portions of petroleum ether containing, first, increasing concentrations of acetone (0.5 to 30%), and then increasing concentrations of methanol (1–4%). The more concentrated fractions from the column were washed with water to remove polar solvents; the more dilute fractions were dried under nitrogen and redissolved in petroleum ether or methanol, depending on their solubility.

Spectra were measured in a Carey recording spectrophotometer. In the course of the work it became apparent that many of the pigments resembled known carotenoids in their spectra except that the observed maxima were a few millimicrons low. A sample of lycopene, prepared from commercial tomato paste (2), had absorption maxima in petroleum ether at 439, 467, and 498 mμ; after isomerization with iodine the peaks were at 437, 465, and 495 mμ. The maxima reported by Zechmeister *et al.* (23) were at 443, 473, 504 and 441.5, 471, and 502 respectively. In ethanol the maxima agreed with those reported by Karrer (9). All maxima reported in petroleum ether in this paper have therefore been adjusted by adding 5 mμ to the values measured.

Iodine isomerizations were carried out by a slight modification of the method of Wright *et al.* (22), the main difference being that petroleum ether was used as a solvent rather than hexane.

To determine the effects of growth conditions on pigment production by red halophiles, cultures were grown in 30-ml aliquots of medium in 250-ml Erlenmeyer flasks on a shaker. The optical density of the suspension was measured at 680 mμ using uninoculated medium as a blank. The cells were then removed by centrifugation, their pigment extracted by treatment with water and acetone, the solution was filtered and diluted to 6.0 ml with acetone, and the optical density measured at 500 mμ. The ratio of the two optical densities was taken as a measure of the degree of pigmentation of the cells. A Coleman Junior spectrophotometer was used in these measurements.

Results

Pigments of the Yellow Halophiles

The yellow coccus, SE 20-4, contained two main pigment types. The first was eluted as a broad band by mixtures of petroleum ether and 5 to 20% acetone. The absorption spectrum of this fraction had maxima at 415, 437, and

468 mμ and closely resembles the spectrum reported by Haxo (7) for neurosporene. Isomerization with iodine caused the maxima to shift downward about 1 mμ and to become less sharp. The breadth of the band suggests that this fraction consists of more than one pigment, possibly neurosporene with one or more of its isomers and one or more of its hydroxylated derivatives.

The second main pigment began to elute with 20% acetone and elution was completed by 2% methanol. Its spectrum showed a plateau at 418–425 mμ and peaks of almost equal intensity at 450 and 485 mμ. Since it distributed itself about equally between petroleum ether and 95% methanol, it is probably a monohydroxy carotenoid but its identity is not known.

Traces of several other pigments which could not be identified were sometimes found. One, which appeared occasionally before the first main pigment, eluted with about 0.5% acetone and had peaks at about 381, 402, and 428 mμ. An epiphasic pigment, with a spectrum with maxima at 443, 468, and 504 mμ, resembling that of lycopene, was sometimes found between the two main fractions. A very strongly absorbed pigment, possibly an oxidative artifact, sometimes remained at the top of the column and could not be eluted even with 100% methanol. It was removed with a mixture of 9 parts methanol and 1 part 1:1 HCl and had peaks in methanol at 400, 425, and 460 mμ.

Coccus H5B-2 contained only one main pigment type which, on the basis of its absorption spectrum and behavior on the column, appeared to be identical with the neurosporene-like fraction of SE 20-4. A trace of another pigment, with absorption peaks at 377, 397, and 427 mμ, was eluted with 1–2% methanol in petroleum ether. It was hypophasic in 95% methanol and may be a hydroxylated derivative of ζ-carotene or θ-carotene.

Pigments of the Red Halophiles

The red halophiles contained a more complex mixture of carotenoids than did the yellow organisms, but the mixture was easier to resolve by chromatography (Table I). Only slight differences were observed amongst the four species studied. Each of the pigments listed was found at least once in *H. salinarium* and *H. halobium*, and all except IV at least once in *H. cutirubrum*. Only one batch of *S. littoralis* was studied and it lacked pigments II and III.

Each of the eight pigments was found in the petroleum ether soluble fraction, although not all of them were found in every lot. The petroleum ether insoluble fraction consisted almost exclusively of pigment VIII, α-bacterioruberin, the main pigment of the red halophiles, although traces of VI and VII were also present. A sample of pigment VIII, purified by chromatography and reprecipitated from a methanol – petroleum ether mixture by dilution with water, contained no detectable methoxyl.*

Biochemistry of α-Bacterioruberin

α-Bacterioruberin does not occur free in the cells but appears to be rather firmly bound to protein. When the cells are lysed by exposing them to low salt concentrations, an almost clear red solution is obtained from which the

*I am grateful to Mr. A. E. Castagne for this analysis.

TABLE I

Carotenoids found in red halophiles

Pigment	Absorption maxima,* mμ	Eluent, petroleum ether containing:	Partition behavior between methanol and petroleum ether	Similar pigment in *Rhodospirillum rubrum*	Remarks
I	425, 449, 475	0–1/2% acetone	Does not enter 95% methanol	G (6), P450(15)	
II	391	1–2% acetone	Distributes between 95% methanol and petroleum ether	—	Gives a blue color with conc. HCl
III	446, 471, 503	2–3% acetone	Does not enter 95% methanol	Lycopene (15)	Traces only (not always detected)
IV	413, 437, 465	5% acetone	Does not enter 95% methanol	B(6), OH-neurosporene(15)	Traces only (not always detected)
V	425, 449, 475	10% acetone	Distributes between 95% methanol and petroleum ether	—	Probably a monohydroxy derivative of I
VI	455, 485, 517	30% acetone (started) 1% methanol (completed)	Enters 95% methanol but not 90%	OH-P481(15)	Slightly less hypophasic than OH-P481. Possibly demethylated P481
VII	455, 482, 514	1–2% methanol	Enters 90% methanol	—	
VIII	462, 493, 524	3–4% methanol	Enters 90% methanol	—	Main pigment; α-bacterioruberin; demethylated spirilloxanthin

*The spectra of VII and VIII were determined in methanol, the others in petroleum ether.

pigment cannot be extracted by non-polar solvents. The same is true of cell extracts prepared by sonic disintegration. When such cell extracts are heated, the pigment remains attached to the precipitated protein. The pigment–protein complex is, however, split when the protein is precipitated by polar organic solvents.

The absorption spectrum of the pigment–protein complex has maxima at rather longer wave lengths than has the spectrum of the free pigment in methanol. The spectra of cells of a large number of strains, lysed in water, had maxima at 475, 505, and 540 mμ. These are rather close to the maxima of the carotenoid part of the spectrum of the spirilloxanthin–chlorophyll–protein complex of *Rhodospirillum rubrum*, i.e., 480, 510, and 550 mμ (3).

The pigment content of the organisms was little influenced by growth conditions. Growing the cells in still rather than shake cultures or alteration of the salt concentration of the medium or of growth temperature had little effect on the degree of pigmentation. Klebahn (12) also found that the production of pigment was unaffected by the physical and chemical conditions of growth.

TABLE II

Influence of age of culture on carotenoid content of *Halobacterium salinarium*

Age of culture, days	$O.D._{680}$ of culture	$O.D._{500}$ of pigments in acetone	$\frac{O.D._{500}}{O.D._{680}}$
4	.200	.355	1.77
7	.264	.599	2.27
10	.328	.768	2.34
12	.300	.785	2.62

Pigmentation was not increased by the addition of carotenoid precursors such as leucine, glycerol, sodium lactate, sodium acetate, or mevalonic acid. The amount of pigment did increase with the age of the culture (Table II). The effect of diphenylamine, which inhibits carotenogenesis in many organisms, could not be determined as it inhibited the growth of the red halophiles.

Discussion

Yellow carotenoids, probably neurosporene or its hydroxy derivatives, are quite commonly found in yellow cocci (5), and H5B-2, therefore, is not unusual in this respect. The pigment of coccus SE20-4 with its main peak at 450 mμ cannot be identified with any known pigment and it is not the same as either of the pigments of the red halophiles with peaks at 450 mμ.

The pigments of the red halophiles are remarkably similar to those of *Rhodospirillum rubrum* (6, 15). Pigment I resembles P450 (so designated from the position of its main absorption peak in petroleum ether) very closely, but it is impossible to suggest the relationship between them until more is known of the structure of P450 and particularly whether or not it is methylated. Only pigment II seems to possess a chromophore not found in any of the pigments of *R. rubrum*. However, it is probably a carotenoid because it gives a blue color with concentrated HCl, and it may be a keto-carotenoid since certain compounds of this type have spectra with single peaks (9). Pigments III and IV were present in minute amounts, but they seem to have chromophores very similar to those of lycopene and neurosporene respectively. Pigment IV is probably a hydroxy compound rather than a hydrocarbon as it is eluted from the column after the lycopene-like pigment III, whereas neurosporene is eluted before lycopene (15). Pigment V, which is probably a hydroxy derivative of pigment I, does not seem to correspond to any of the pigments found in *R. rubrum*. Either pigment I or pigment V is probably identical with the pigment having its main peak at 450 reported by Spruit and Pijper (18). Pigment VI has a spectrum similar to P481 and hydroxy-P 481 (main absorption maxima at 481 in petroleum ether), but seems to be intermediate between them in its partition behavior between petroleum ether and methanol. P481 contains one methoxyl group (14); if it were demethylated the resulting monohydroxy compound would probably be very similar to pigment VI. Pigment VII might be either a dihydroxy analogue of VI, or a stereoisomer of VIII. Either VI or VII probably corresponds to Petter's β-bacterioruberin. Pigment VIII is undoubtedly Petter's α-bacterioruberin, and the absence of methoxyl supports Lederer's suggestion that this compound is demethylated spirilloxanthin. It is not identical with the OH-spirilloxanthin of *R. rubrum*, which is less hypophasic than VIII.

From an analysis of the kinetics of the synthesis of carotenoids in cells previously inhibited with diphenylamine, Liaaen Jensen *et al.* (15) concluded that the synthesis of the chromophore of spirilloxanthin in *R. rubrum* follows the sequence: phytoene $\xrightarrow{?}$ phytofluene$\rightarrow$ ζ-carotene $\rightarrow$ neurosporene $\rightarrow$ lycopene $\rightarrow$ P481 $\rightarrow$ spirilloxanthin. The relationship between the various hydroxyl-

ated carotenoids and the corresponding hydrocarbons was not determined and it is not known exactly when the carotenoid chain is methylated, although methoxyl groups are found as early in the sequence as P481 (14).

The present results suggest that many of the carotenoids of the red halophiles are identical with pigments found in *R. rubrum*, or are demethylated derivatives of them. It seems likely, therefore, that the pathway of carotenoid biosynthesis in red halophiles is the same as in *R. rubrum*, except that no methylations occur.

It is interesting that only minor differences were observed in the carotenoids of the four organisms studied, particularly since they differ considerably in the appearance of their colonies on agar. *H. salinarium* is a fairly bright scarlet while *H. cutirubrum* has a purplish tinge. *H. halobium* is a very pale pink. *Sarcina littoralis* is more orange than the *Halobacterium* species. The appearance of *H. halobium* is due to the presence of gas vacuoles (8, 17) and it is likely that the variations in hue among the other strains are due to physical rather than chemical differences.

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THE COMPONENT FATTY ACIDS OF OILS FOUND IN SPORES OF PLANT RUSTS AND OTHER FUNGI[1]

A. P. TULLOCH AND G. A. LEDINGHAM

Abstract

The fatty acid compositions of oils extracted from the spores of 21 species of fungi, mainly rusts and smuts, have been determined by gas liquid phase chromatography. *Cis*-9,10-epoxyoctadecanoic acid was present in the spore oil of six species of *Puccinia*, two species of *Melampsora*, and one species each of *Cronartium* and *Phragmidium*, but not in one species each of *Uromyces*, *Gymnosporangium*, and *Ravenelia*. Palmitic acid was the principal saturated acid in all the fats, except those from the mildews, and the unsaturated C_{18} acids generally made up the remainder. Rusts of the same genus with hosts in the same plant family had similar fatty acids but other rusts of the same genus with hosts in other families had a quite different pattern. The fats of smuts and mildews are quite distinct from those found in the rusts.

Introduction

Very little is known of the composition of the fats present in the spores of fungi though various investigations were carried out by Zellner and co-workers in 1904–1935 (11) but they used older methods and at best could do no more than determine the principal acids present. Ergot oil has been analyzed by modern methods (1) but in most cases, particularly with fungi such as rusts and mildews which cannot be grown on synthetic media, the amount of material obtainable is much too small for the conventional type of analysis. Now, however, it is possible to analyze very small samples of oil rapidly and accurately by means of gas liquid phase chromatography.

Previous papers (13, 14) have reported the fatty acid composition and the isolation of *cis*-9,10-epoxyoctadecanoic acid from the oil of the uredospores of *Puccinia graminis* var. *tritici* race 15B. The present paper describes the analysis of the spore oils of some cereal rusts, various other rusts on different hosts, and a number of smuts and mildews particularly with regard to the amount of epoxy acid present.

Materials and Methods

Uredospores of *Puccinia graminis avenae* race 7A and of *P. triticina* were obtained from Dr. G. J. Green of the Canada Department of Agriculture Laboratory in Winnipeg. The following materials were all collected locally: uredospores of *P. graminis tritici* race 56 and of *P. coronata*; teliospores of *P. carthami* on *Carthamus tinctoris*; *Melampsora medusae* uredospores on *Populus balsamifera*; *Uromyces psoraleae* aeciospores on *Psoralea argophylla*; dried gelatinous horns containing teliospores of *Gymnosporangium juvenescens* on *Juniperus horizontalis*; conidia of *Erysiphe graminis* on Stewart wheat

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and of *Sphaerotheca humili* var. *fuliginia* on *Taraxacum officinale*. These spores were collected by suction; later it was found that spores could be collected more rapidly by swirling the infected plant material in petroleum ether, b.p. 40–60° C, for a few seconds, after which the dislodged spores were filtered and sieved. This method was used for the uredospores and teliospores of *P. hieracii* on *T. officinale*, for the teliospores of *P. helianthi* on *Helianthus annuus*, and for the teliospores of *Phragmidium speciosum* on *Rosa woodsii*. The uredospores of *P. hieracii* collected in this way were able to reinfect dandelion plants. The uredospores of *M. lini* were grown on Redwing flax in the greenhouse. The aeciospores of *Cronartium harknessii* were obtained from Dr. J. C. Hopkins of the Forest Biology Laboratory, Calgary, Alberta. The chlamydospores of *Ustilago zeae* were from Dr. B. C. Jenkins, University of Manitoba, Winnipeg, and chlamydospores of *U. tritici* on Lee wheat, *U. nigra* on barley, *U. levis* on oats, and *Tilletia foetens* on wheat were obtained from Dr. R. C. Russell of the Canada Agriculture Research Laboratory, Saskatoon. Teliospores of *Ravenelia hobsoni* were collected on *Pongamia glabra* by Dr. M. J. Thirumalachar at Poona, India.

The oil was extracted by grinding the spores overnight in a ball mill with ethyl ether, which was removed under vacuum, and the oil stored at −20° C.

Preparation of the Methyl Esters

The oil (0.3 g) was treated with a small amount of ethereal diazomethane to esterify any free acid present which might react with the catalyst and refluxed with 0.02 *N* methanolic sodium methoxide (5 ml) for 1 hour and poured off from insoluble material. The catalyst was neutralized with a few drops of acetic acid, the alcohol removed under vacuum, the residue taken up in ether, washed with water, dried over sodium sulphate, and the ether taken off under reduced pressure. The proportion of non-saponifiable matter was not measured when esters were prepared in this way, but in some cases when sufficient material was available it was determined after saponification. The percentages of fatty acids reported are percentages of the total fatty acids present, not of the whole oil.

Determination of Composition

The methyl esters were analyzed by gas liquid phase chromatography (G.L.P.C.) using both silicone and ethylene glycol succinate polyester columns (13), and good agreement was obtained between the two sets of results. The apparatus used was described in previous publications (4, 5). A 6-ft × $\frac{1}{4}$-in. column of silicone grease on Celite was prepared and operated as before (13). The polyester column was made up with ethylene glycol succinate on acid-washed Celite, 80–100 mesh, in the ratio of 1:6 w/w, and a 6-ft × $\frac{1}{4}$-in. copper column was used. The unit was operated at 215° C with injector at 250° C and a flow rate of 40–60 ml helium per minute. The chain length and degree of unsaturation of the components were deduced from their emergence times. The presence of epoxy acid was suggested by the appearance of a peak with different emergence times on the two columns (13), at the C_{20} position on the silicone and at about the C_{25} saturated position on the polyester column. In

all oils where epoxy acid was indicated in this way, further evidence for its presence was obtained by refluxing the oil with glacial acetic acid for a few hours and analyzing on the silicone column when the peak at the C_{20} position had disappeared and an acetoxy–hydroxyester peak at about the C_{23} position had appeared (13). When enough oil was available, the product after acetolysis was saponified, and *threo*-9,10-dihydroxyoctadecanoic acid, produced from the epoxy acid, was isolated by distribution between petroleum, b.p. 40–60° C, and 80% methanol in five separatory funnels. The two most polar fractions were combined and crystallized from acetone at 0° C; the yield of dihydroxy acid was in good agreement with the amount expected from the percentage of epoxy acid in the oil. The acid had m.p. 92–94° C and did not depress the melting point of authentic *threo*-9,10-dihydroxyoctadecanoic acid (13).

Oxidation of the Acids

The unsaturated C_{18} acids most commonly found in natural fats are oleic, linoleic, and linolenic acids in all of which the unsaturation commences at the ninth carbon atom. Though these acids are well separated by the polyester column (4, 5), they cannot be distinguished with certainty in gas liquid chromatographic analysis from isomeric unsaturated acids which might possibly be present, for example 6-octadecenoic acid which is found in umbelliferous seed oils (6). However, if the fatty acids are oxidized with the permanganate–periodate reagent (8, 9, 10), azelaic acid is the sole dicarboxylic acid produced when only the common unsaturated C_{18} acids are present. Conjugated unsaturated acids could also yield azelaic acid but they can be detected by their characteristic ultraviolet absorption spectra. Comparison of the relative amounts of azelaic acid produced and the palmitic and stearic acids with that calculated, assuming that only the common C_{18} acids are present, provides further evidence for the structure of the component fatty acids. Where the amount of oil available was too small for oxidation, it was assumed for the time being that only the usual C_{18} acids were present.

The acids, after separation from epoxy acid if present, were oxidized in aqueous solution and the products isolated and converted to methyl esters. The mixture was analyzed by G.L.P.C. using a polyester column made up with butanediol succinate on 60–80 mesh acid-washed C_{22} firebrick in a ratio of 1:6 w/w. An 8-ft × 3/16 in. copper column was used and the operating conditions were the same as for the other polyester column.

Experimental Results

The fatty acids present in the oil of the uredospores of stem rust of wheat and oats, leaf rust of wheat, and crown rust of oats are shown in Table I. Epoxy acid was removed from the oil of wheat stem rust race 56 by countercurrent distribution and characterized as the dihydroxy acid. The remaining acids were oxidized giving the products below.

	Myristic	C_{15}	Palmitic	Stearic	Azelaic	Suberic
Found, %	4.0	1.1	68.8	5.6	17.8	2.7
Calculated, %	3.9	.7	68.3	4.5	22.6	

TABLE I

The component acids of uredospore oils of cereal rusts

	Rust			
Fatty acid	*Puccinia graminis* var. *tritici* race 56	*Puccinia graminis avenae* race 7A	*Puccinia tritilcina* mixed race	*Puccinia coronata*
	Percentage of fatty acid			
Lauric	0.2	—	.1	—
Myristic	2.2	1.9	2.1	2.5
Pentadecanoic	.3	.2	.3	.5
Palmitic	41.3	35.5	38.1	52.5
Palmitoleic	.4	.5	.8	1.0
Stearic	4.8	6.0	4.5	6.4
Oleic	16.7	11.4	9.5	8.0
Linoleic	4.4	6.1	4.1	4.1
Linolenic	10.4	8.0	6.6	2.3
Eicosenoic	—	—	1.6	—
Behenic	0.3	.9	1.0	.8
Cis-9,10-epoxyoctadecanoic	19.0*	29.5	31.3	21.9
Oil content, %	18	16	17	17

*Some samples also contained about 5.0–11.0% of (+) *threo*-9,10-dihydroxyoctadecanoic acid.

Since azelaic acid was the principal dicarboxylic acid produced, it was assumed that only the common C_{18} acids were present. Though identification of the epoxy acid and oxidation were carried out for one oil only, the compositions of all four are sufficiently similar that the same acids may be assumed to be present in all of them.

Table II shows the composition of the spore oil of three rusts parasitic on members of the family Compositae.

Epoxy acid was characterized as dihydroxy acid for the oils of *P. hieracii* uredospores and *P. carthami* teliospores. The other acids of the oil of *P. hieracii* uredospores were oxidized with the result below.

	Monocarboxylic acids					Dicarboxylic acids		
	C_{14}	C_{15}	C_{16}	C_{18}	C_{22}	C_7	C_8	C_9
Found, %	.6	.7	23.7	7.7	.9	.8	3.2	62.4
Calculated, %	.5	.5	22.7	7.2	1.3			67.8

Again the results indicated that only the common C_{18} acids were present. The ultraviolet absorption spectrum of the fatty acids of the uredospore oil showed no conjugated diene or triene acids. The oil was much more viscous than would have been expected from its composition and the fatty acids obtained after separation of the non-saponifiable material (7.6%) and the epoxy acid gelled at room temperature as did the methyl esters obtained after oxidation. A mixture of weighed amounts of the fatty acid esters and methyl eicosenoate as marker was analyzed by G.L.P.C. and the result showed that a substance forming about 5% of the esters was not being detected. This material which is apparently not susceptible to oxidation may be the cause of the high viscosities observed.

TABLE II

The component acids of spore oils of rusts on Compositae

Fatty acid	Rust and spore type			
	Puccinia hieracii uredospore	*Puccinia hieracii* teliospore	*Puccinia helianthi* teliospore	*Puccinia carthami* teliospore
	Percentage of fatty acid			
Myristic	.3	.3	1.0	.4
Pentadecanoic	.4	.4	.9	.4
Palmitic	16.3	17.1	23.3	17.5
Palmitoleic	.5	.3	1.3	.5
Stearic	5.5	4.6	4.1	3.2
Oleic	11.7	15.1	15.0	28.4
Linoleic	21.1	19.2	11.9	22.2
Linolenic	33.7	34.0	23.0	22.6
Behenic	1.0	.5	.3	—
Cis-9,10-epoxyoctadecanoic	9.5	8.5	16.4	4.8
Unidentified	—	—	2.8	—
Oil content, %	8.4	7.3	13.3	9.0

Tables III and IV show the composition of the spore oils of other rusts. Epoxy acid was characterized as dihydroxy acid for both species of *Melampsora*. The acids of *M. medusae* spore oil, after separation of non-saponifiable material (10.5%), were oxidized with the result below, which showed that largely the usual acids were present.

	Monocarboxylic acids						
	C_{14}	C_{15}	C_{16}	C_{17}	C_{18}	C_{20}	C_{22}
Found, %	3.6	1.4	51.3	.6	10.4	1.5	2.2
Calculated, %	4.1	1.5	54.2	.7	10.0	.5	1.5

	Dicarboxylic acids					
	C_6	C_7	C_8	C_9	C_{10}	C_{11}
Found, %	1.8	1.0	2.9	21.9	.4	1.0
Calculated, %				27.5		

Table V shows the composition of the oil of the chlamydospores of five cereal smuts.

The acids of *Tilletia foetens* spore oil were separated from non-saponifiable material (5%) and oxidized.

	Monocarboxylic acids			Dicarboxylic acids				
	C_{14}	C_{16}	C_{18}	C_7	C_8	C_9	C_{11}	C_{13}
Found, %	.5	22.4	1.4	.7	2.3	66.1	4.8	1.8
Calculated, %	.7	20.1	1.3			77.9		

The C_{18} acids were mainly the common acids.

Evidence for the Structure of the Major Acids of Ustilago zeae Spore Oil

Methyl esters (2.0 g) of *U. zeae* spore oil were separated into C_{16}, C_{18}, and C_{20} fractions in 40-mg batches using the 6-ft silicone column and the weights of the fractions obtained were in good agreement with those calculated by G.L.P.C.

TABLE III

The component acids of rust spore oils

	Rust and spore type			
	Phragmidium speciosum teliospore	*Cronartium harknessii* aeciospore	*Melampsora lini* uredospore	*Melampsora medusae* uredospore
Fatty acid	Percentage of fatty acid			
Myristic	.4	1.1	.6	1.3
Pentadecanoic	—	.8	.4	.6
Palmitic	10.4	17.7	11.1	32.0
Palmitoleic	.6	1.2	.3	1.3
Heptadecanoic	—	—	—	.4
Stearic	3.0	4.8	3.1	5.7
Oleic	14.7	6.9	2.2	7.0
Linoleic	20.1	5.2	2.0	6.7
Linolenic	42.3	12.1	3.0	7.8
Arachidic	.4	.5	1.1	.7
Eicosenoic	—	1.6	.6	—
Behenic	.7	1.0	1.4	1.2
Cis-9,10-epoxyoctadecanoic	7.5	38.0	74.2	30.8
Unidentified	—	(5) 9.1	—	(2) 4.5
Oil content, %	9.5	12.5	14.6	14.6

TABLE IV

The component acids of rust spore oils

	Rust and spore type		
	Uromyces psoraleae aeciospores	*Ravenelia hobsoni* teliospores	*Gymnosporangium juvenescens* dried gelatinous horns
Fatty acid	Percentage of fatty acid		
Myristic	.9	.8	.6
Pentadecanoic	.3	.6	.7
Palmitic	19.9	23.7	11.6
Palmitoleic	1.4	1.6	1.0
Stearic	8.8	9.2	9.6
Oleic	40.9	46.1	2.9
Linoleic	5.2	6.4	7.7
Linolenic	15.6	3.0	54.6
Arachidic	—	1.8	2.9
Behenic	1.1	1.9	3.3
Unidentified	(5) 5.9	(2) 4.9	(2) 5.1
Oil content, %	10.2	18.8	6.3

Palmitic and 9-Hexadecenoic Acids

The C_{16} fraction (0.265 g) was hydroxylated with hydrogen peroxide and formic acid (12) and the mixed acids obtained on saponification stirred with petroleum, b.p. 40–60° C. The soluble portion (0.120 g), after crystallization from methanol, melted at 61–62° C and did not depress the melting point of authentic palmitic acid. The insoluble portion (0.108 g), after crystallization from ethyl acetate, had m.p. 86.5–87.5° C, reported for *threo*-9,10-dihydroxyhexadecanoic acid 85–86° C (2). Permanganate–periodate oxidation of the dihydroxy acid gave azelaic acid, m.p. 105–107.5° C, which did not depress that of an authentic sample.

TABLE V

The component acids of the chlamydospore oil of cereal smuts

Fatty acid	Smut				
	Tilletia foetens	*Ustilago zeae*	*Ustilago tritici*	*Ustilago nigra*	*Ustilago levis*
	Percentage of fatty acid				
Myristic	.5	.4	.3	1.4	1.1
Pentadecanoic	—	.2	2.7	1.2	.9
Palmitic	14.6	9.3	26.9	24.9	19.8
9-Hexadecenoic	3.0	5.2	9.7	5.7	6.8
C_{16} with two double bonds	1.2	—	—	—	—
Stearic	1.0	2.6	4.7	5.5	3.9
Oleic	8.8	53.8	30.2	35.0	42.0
Linoleic	63.2	17.4	16.4	22.0	19.0
Linolenic	—	—	—	1.6	1.6
Arachidic	.3	2.3	1.0	.5	—
9-Eicosenoic	1.2	7.6	1.9	1.0	3.2
Behenic	.4	1.2	—	.4	.2
Unidentified	(2) 6.0	—	3.5	.8	1.5
Oil content, %	20	22	5	4	14.5

The C_{18} Acids

The acids of the C_{18} fraction on oxidation gave:

	Stearic	Dicarboxylic acids		
		C_8	C_9	C_{11}
Found, %	4.5	4.8	88.0	2.7
Calculated, %	3.7		96.3	

The principal unsaturated acids are mainly oleic and linoleic acids.

Arachidic and 9-Eicosenoic Acids

The C_{20} fraction (0.207 g) was hydroxylated and treated in the same way as the C_{16} fraction. The petroleum-soluble portion (0.058 g) was esterified with diazomethane and when repurified by G.L.P.C. gave methyl arachidate, m.p. 44–45° C, reported m.p. 46.6° C. The petroleum-insoluble portion gave, after crystallization from ethyl acetate, a dihydroxyarachidic acid (0.095 g), m.p. 97–98° C, which depressed the melting point of *threo*-11,12-dihydroxyarachidic acid (m.p. 96–98° C, prepared from 11-eicosenoic acid isolated from rapeseed oil) to 86–98° C; reported melting point for *threo*-9,10-dihydroxyarachidic acid is 97–98° C (2). Oxidation of the dihydroxy acid gave azelaic acid, m.p. 103–106° C, undepressed by an authentic sample.

Table VI gives the composition of the oil of the conidia of two powdery mildews.

As poor yields were obtained on methanolysis, the methyl esters of both oils were prepared by saponification, isolation of the acids, and esterification with diazomethane. The unidentified components of the oil of *Erysiphe graminis* had emergence times in the C_{23}–C_{24} and C_{25}–C_{26} regions using both silicone and polyester columns which suggests that they are not oxygenated acids; they may be branched chain acids or acids containing cyclic systems. The amount of oil available was insufficient for further characterization.

TABLE VI

The component acids of the oil of conidia of mildews

	Mildew	
	Sphaerotheca humili var. *fuliginia*	*Erysiphe graminis*
Fatty acid	Percentage of fatty acid	
Lauric	.2	—
Myristic	1.1	.8
Pentadecanoic	—	.3
Palmitic	5.3	8.8
Palmitoleic	1.0	1.0
Stearic	2.2	2.0
Oleic	4.5	4.3
Linoleic	7.0	6.0
Linolenic	4.3	5.1
Arachidic	13.0	.8
Behenic	41.7	5.0
C_{22} with one double bond	1.7	7.1
Lignoceric	10.0	7.6
C_{24} with one double bond	—	6.5
Unidentified	(2) 8.0	(7) 44.7
Non-saponifiable, %	26	30
Oil content, %	10	12

Discussion

The epoxy acid is not peculiar to the spore fats of fungi as, while this work was in progress, it was reported to be a minor constituent of the seed oil of *Tragopogon porrifolius* (3).

Some preliminary conclusions can be reached concerning the compositions of the spore fats of the *Puccinia* species. It was hoped that the fat analysis might be useful in distinguishing between the various biological and physiological races of *P. graminis* but as can be seen from Table I, the compositions are all very similar to that of race 15B of wheat stem rust (13). Palmitic acid is the major acid and moderate amounts of each of the four common C_{18} acids, together with the epoxy acid, make up the remainder. In general the differences are not sufficiently large to be useful taxonomically, though the higher percentage of oleic acid in the spore oil of race 56 of wheat stem rust may be characteristic. The presence of an appreciable proportion of dihydroxy acid in some other samples of this oil is probably not significant as the spores were 9 years old and there is some reason to believe that epoxy acid is converted in the spore to dihydroxy acid during long storage.

Table II shows that the spore oils of the rusts on dandelion, sunflowers, and safflowers, where the hosts are all in the family Compositae, have a similar composition which is quite different from that of the *Puccinia* species on cereals. Here the principal acids are the unsaturated C_{18} acids and the epoxy acid is a lesser component (5–16%). It is interesting that the oils of uredospores and teliospores of *P. hieracii* are almost identical in composition suggesting that the rust produces only one type of oil, at least on one host.

The differences in oil composition found when rusts of the same genus are on hosts of different plant families is again shown in Table III. The epoxy acid is present in the uredospore oil of *Melampsora lini* on flax in the remarkably high proportion of 74% but oil of *M. medusae* on poplar trees also has palmitic and epoxy acids as major components and an appreciable proportion of C_{18} acids. The other two rusts in this table had a moderate epoxy acid content.

The epoxy acid was not found in the oil of the dried gelatinous horns of *Gymnosporangium juvenescens* (Table IV). The spore oils of *Uromyces psoraleae* and *Ravenelia hobsoni*, both on Leguminosae, which had quite similar compositions, contained about 2% of a compound which appeared to be some type of oxygenated acid but which was probably not the epoxy acid. Other members of these genera will have to be examined before this can be considered characteristic.

The composition of the chlamydospore oil of the cereal smuts (Table V) is quite different from that of the cereal rusts and has several points of interest. First, epoxy acid is absent, and secondly, an appreciable proportion (3–10%) of 9-hexadecenoic acid, commonly known as palmitoleic acid, is present. This acid is a regular component of many marine animal oils but very rarely forms more than 1% of the seed oil of higher plants. Thirdly, *U. zeae* spore oil contains about 8% (less in the others) of 9-eicosenoic acid which has previously been found only in certain fish oils (7). Oleic acid is the major component of the oils of the *Ustilago* species accompanied by a considerable proportion of linoleic acid. The spore oil of *Tilletia foetens*, the stinking smut of wheat, differs in that the characteristic acid is linoleic acid.

Finally, the oils of the conidia of the powdery mildews on dandelions and on wheat (Table VI) are remarkable for the presence of a major proportion of long-chain acids. Behenic acid is the principal acid of the former and while a large percentage of the acids of the latter could not be identified, they are most probably longer than C_{22}; the content of C_{16} and C_{18} acids of the two oils is very similar. The difficulties of preparing the esters and the large amount of non-saponifiable material suggests that they may not be simple triglycerides.

This study is being continued with other rusts and possible relatives of the Uredinales. It is hoped that it may be possible to compare the fats of all the spore forms present in several autoecious and heteroecious rusts in order to assess the role played by the host in determining fatty acid type and distribution in these obligate parasites.

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LA PRODUCTION DES ENZYMES PECTIQUES PAR CORYNEBACTERIUM SEPEDONICUM[1]

ROGER PAQUIN, ROBERT-A. LACHANCE ET LOUIS-J. COULOMBE

Abstract

Corynebacterium sepedonicum growing on synthetic media containing various pectic substances did not produce any of the following pectic enzymes, pectin methylesterase, depolymerase, polygalacturonase, and protopectinase, even with the addition of glucose. Moreover, none of these enzymes was found in potatoes heavily contaminated with *C. sepedonicum* and showing symptoms of ring rot.

Introduction

Bien que le rôle des enzymes pectiques dans le rouissage du lin soit connu depuis plus d'un siècle, la découverte de leur sécrétion par les microorganismes pathogènes et leur rôle dans les maladies des plantes est d'origine beaucoup plus récente. Le mécanisme de leur action est étudié de façon assez intensive depuis 10 ans à peine et nous ignorons encore beaucoup de choses à leur sujet.

Stapp (11) fait mention dans un tableau reproduit par Matus (7) et Kertesz (6) de la production d'une polygalacturonase (PG) par *Corynebacterium sepedonicum*, agent responsable de la flétrissure bactérienne de la pomme de terre. Plus récemment, Smith (10) a étudié la production des enzymes pectiques par 67 espèces de bactéries et aucune espèce du genre *Corynebacterium* telles que *C. fascians*, *C. flaccumfaciens* et *C. michiganense* n'a produit de pectine glycosidase (polygalacturonase), ni de pectine méthylestérase (PME).

Le but du présent travail était donc de déterminer si *C. sepedonicum* produit des enzymes pectiques et de jeter quelque lumière sur le mode d'action de cet agent pathogène dans les tissus de son hôte.

Matériel et méthodes

Le milieu synthétique de base auquel furent ajoutés différents hydrates de carbone avait la composition suivante: .05% de K_2HPO_4 et KH_2PO_4, .02% de $MgSO_4.7H_2O$, .001% de NaCl, $FeSO_4.7H_2O$ et $MnSO_4.4H_2O$, 0.5% de caséine hydrolysée, 2 mg/l. d'acide nicotinique, 0.6 mg/l. de thiamine et 2 μg/l. de biotine. Le pH des milieux fut ajusté à 6.7–6.8. Après la stérilisation, ils furent inoculés avec une goutte d'une forte suspension de bactéries provenant de deux souches de *Corynebacterium sepedonicum* isolées dans nos laboratoires.

La croissance bactérienne fut suivie en mesurant la turbidité des milieux avec un colorimètre (Spectronic 20 de Bausch and Lomb), à 600 mμ. Des milieux de même composition, non inoculés, servirent de témoins.

La méthode d'Elarosi (2) utilisée pour l'inoculation d'un milieu naturel tel que la pomme de terre fut modifiée comme suit afin d'éviter les contaminations: les cylindres de pommes de terre de 18 mm de diamètre et de 25 à 30 mm de

[1]Manuscrit reçu le 7 avril 1960.
Contribution du Laboratoire des Recherches, Ministère de l'Agriculture du Canada, Ste-Anne-de-la-Pocatière, Qué.

long, obtenus à l'aide d'un emporte-pièce désinfecté, furent plongés dans l'alcool à 70%, et immédiatement placés dans des flacons préalablement stérilisés. La stérilisation des cylindres, leur inoculation, de même que l'extraction et la purification des enzymes se firent suivant la méthode d'Elarosi (2).

La présence dans le milieu de culture de la pectine méthylestérase (PME) fut déterminée par réaction avec l'hydroxylamine sur couche d'agar (8), et sa concentration par titration des groupes carboxyles libérés avec l'hydroxide de sodium (NaOH, $N/50$) suivant la méthode de Kertesz (6) modifiée par Kapica (5).

La méthode de Wood (12) fut utilisée pour déterminer l'activité de la dépolymérase (DP). Les résultats exprimés sont en pourcentage de perte de viscosité relative durant les trois premières minutes de la réaction (9).

La production de l'acide monogalacturonique par l'action de la polygalacturonase (PG) sur les molécules de pectine, fut révélée par chromatographie (12) en utilisant la benzidine comme révélateur, et titrée avec l'iode suivant le procédé de Jansen et MacDonnell (4).

La macération des tissus de pommes de terre par les filtrats des cultures (9, 12) servit à mesurer l'activité de la protopectinase (PP).

Les mêmes méthodes furent utilisées avec les filtrats de *Fusarium lycopersici* qui produit ces enzymes, afin de nous assurer de leur efficacité dans les conditions expérimentales où nous opérions.

Résultats

La figure 1 montre la croissance de *C. sepedonicum* sur des milieux de cultures contenant différentes sources de carbone. La bactérie ne croît pas dans les milieux contenant 0.25 à 1.5% de pectine pure. Elle croît assez bien dans les milieux additionnés de 0.5% de pectate de sodium ou de calcium, et tardivement sur ceux contenant 0.5% d'acide α-D-monogalacturonique. La présence de 0.25% de glucose favorise la croissance de la bactérie dans les milieux contenant 0.25% de pectine ou d'acide α-D-monogalacturonique. Lorsqu'il y a croissance, le pH évolue et passe du côté alkalin indiquant que le milieu est plus ou moins métabolisé.

Nous n'avons pu déceler la moindre trace de PME, de PG, de DP et de PP sur aucun des milieux utilisés. Des essais d'extraction et de purification des enzymes ne donnèrent pas plus de résultats. *C. sepedonicum* semble donc inapte à synthétiser les enzymes pectiques, même en présence d'une double quantité de glucose propre à amorcer la croissance de l'organisme. Cinq ou six repiquages successifs sur un mélange de pectine et de glucose n'ont pas rendu l'organisme plus apte à utiliser la pectine comme source de carbone. A ceci viennent s'ajouter les preuves suivantes: (1) des analyses chimiques nous ont démontré que la quantité de pectine initiale demeurait inchangée après plusieurs jours d'incubation; (2) la viscosité des milieux de culture demeurait pratiquement la même durant toute la période de croissance.

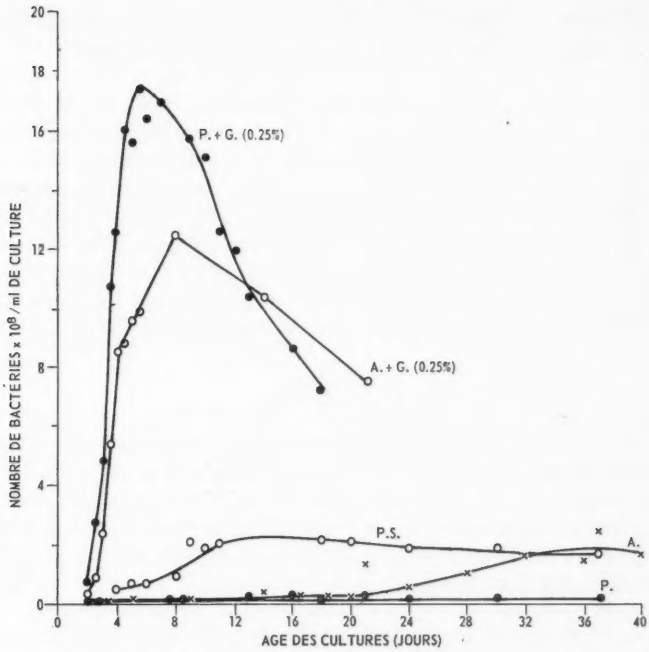

FIG. 1. Croissance de *Corynebacterium sepedonicum* sur des milieux de culture contenant comme sources de carbone, 0.25 à 1.5% de pectine (P.), 0.1 à 0.5% d'acide galacturonique (A.), 0.5% de pectate de sodium (P.S.), 0.25% de pectine additionnée de 0.25% de glucose (P.G.), ou 0.25% d'acide galacturonique additionné de 0.25% de glucose (A.G.).

C. sepedonicum semble capable de métaboliser très lentement l'acide monogalacturonique en l'absence de toute autre source d'hydrate de carbone (Fig. 1). Après plus d'un mois d'incubation, des traces de cet acide étaient encore présentes dans les milieux de culture qui en contenaient 1% et 5% initialement.

C. sepedonicum ne produit pas davantage d'enzymes pectiques dans des milieux naturels tels que les pommes de terre inoculées artificiellement ou atteintes de flétrissure et provenant d'une semence fortement contaminée. Des essais de précipitation et de purification des enzymes n'ont pas donné de meilleurs résultats.

Discussion

La production des enzymes pectiques par certains microorganismes pathogènes tels qu'*Erwinia carotovora*, *E. aroideae*, *Pythium* de *Baryanum* et *Botrytis cinerea*, cause généralement une pourriture rapide des végétaux, provoquant la désintégration des tissus et plus particulièrement celle des membranes cellulaires en attaquant leurs constituants pectiques (1, 3, 10). Rien de tel ne se produit dans les tubercules de pommes de terre envahis par *C. sepedonicum* et gardés à l'abri des contaminations par les organismes secondaires provenant

du sol ou d'autres sources. Il ne faut donc pas se surprendre que *C. sepedonicum* ne produise pas d'enzymes pectiques, le contraire eut été étonnant.

La croissance de la bactérie sur des pectates de sodium et de calcium pourrait peut-être s'expliquer par une action déméthoxylante due au pH des milieux de culture. De même la croissance sur l'acide monogalacturonique trouverait peut-être une explication dans le fait que cet acide est le produit final de l'action catalytique des enzymes pectiques et que sa configuration moléculaire est voisine de celle du glucose. D'autre part, la désintégration très lente des tissus de la zone vasculaire et du parenchyme environnant dans les tubercules atteints de flétrissure nous incite à croire qu'il s'agit ou d'une action mécanique due à la croissance abondante de l'agent pathogène dans les tissus ou d'une action biochimique très faible due à une ou des sécrétions bactériennes.

Résumé

Corynebacterium sepedonicum (Spieck. et Kott.) Skapt. et Burkh., cultivé sur des milieux synthétiques contenant différentes substances pectiques avec ou sans apport de glucose, s'est révélé impuissant à produire des enzymes pectiques telles que la pectine méthylestérase, la dépolymérase, la polygalacturonase et la protopectinase. Aucune trace de ces enzymes ne fut décelée dans les tubercules de pomme de terre inoculés ou provenant d'une semence fortement contaminée.

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A SIMPLE METHOD FOR THE DETERMINATION OF THE RESPIRATORY QUOTIENT OF SOILS[1]

G. STOTZKY[2]

Abstract

A simple method for the determination of the respiratory quotient of soils is described. The advantages of this method over those previously described are: (1) Variability between identical treatments is minimized. (2) Errors arising from non-uniform respiration of replicated treatments are eliminated. (3) Soil moisture contents are maintained at a constant level. (4) R.Q.'s can be correlated directly with microbial activity as indicated by CO_2 evolution and both parameters are collected from a single soil sample for extended periods of time. (5) C^{14}-enriched substrates may be used. (6) The composition of the ambient atmosphere can be easily controlled. A brief comparison of different methods is presented.

Introduction

During an investigation of factors affecting microbial activities of soil, the possibility that anaerobic dissimilation of added glucose was occurring made it necessary to determine the respiratory quotient (R.Q.) of soils amended with glucose. The R.Q. should disclose anaerobiosis as well as possibly suggest the types of intermediates, resulting from glucose decomposition, which serve as substrates for different microbial populations during the course of incubation. The relatively few published reports on the R.Q. of soils have been based on the use of manometric techniques, primarily the "direct" method of Warburg and co-workers (27). This method employs two respirometers: one which contains alkali to absorb evolved CO_2 and gives a measure of O_2 uptake, and one which contains no alkali, the difference in pressure between the two respirometers being a measure of CO_2 evolution (5, 6, 7, 9, 10, 14, 15, 16, 20, 21, 28). A technique wherein O_2 is continually supplied by electrolysis of water and the generated H_2 is measured manometrically after the evolved CO_2 has been absorbed in alkali has recently been used by a number of investigators to determine O_2 consumption (1, 11, 17, 26, 29). This "electrolytic" method, developed by Helvey (13), was first applied to soils by Swaby and Passey (26) and further modified by Birch (1), McGarity *et al.* (17), Wieringa and Mogot (29), and Greenwood and Lees (11).

These techniques have been used, for the most part, to measure the respiratory capacity of soils for short periods of time lasting only from 8 to 12 hours. In our investigations it was necessary to measure the R.Q. during experiments extending from several days to several weeks. Since anaerobiosis was suspected, it was quite probable that gases other than CO_2 and O_2 were involved: e.g., H_2, CH_4, H_2S, volatile nitrogen compounds. The accumulation of these gases in reaction vessels that would be closed for any length of time,

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Paper No. 28 from the Plant Nutrition Project, The University of Michigan, Ann Arbor, Michigan.
[2]Present address: Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

as in the electrolytic method, presented the possibility that microbial activity would be retarded in long-term experiments. The maintenance of the moisture content during incubation was mandatory, since previous experiments with the soil used in this study had shown that fluctuations in soil moisture content were reflected in changes in microbial activity. Loss of moisture from the soil by diffusion into the alkali used for absorption of CO_2 was anticipated, and a method of maintaining the moisture content at a constant level was required. Periodic measurements of CO_2 are not conveniently conducted with the electrolytic type of respirometer and this limitation, plus the requirement for specialized equipment, eliminated this technique. Since changes in the R.Q. were to be correlated with CO_2 evolution curves, it was desired that both these parameters be measured on the same soil sample. An analysis of published procedures, as well as attempts to apply several of these procedures (22), indicated that they were not adequate and required modification in order to fulfill the objectives of our experiments.

Materials and Methods

In order to decrease the variability in the respiration patterns which was observed in preliminary experiments between replicated 4- to 8-g samples of soil incubated in standard volume (15 ml) Warburg respiratory vessels, the use of vessels with a total volume ranging from 90 to 100 ml was investigated. These vessels[3] differed from the conventional type in that they had no center well and were composed of two sections connected by a ground glass joint: a bottom section with a side arm, and a top section which contained the joint by which the vessels were attached to the manometers. The vessels conveniently held 25 to 35 g of soil, but, for ease of calculation, only 25 g of soil was used. There was no effect of sample size on the relative respiratory capacity nor on the R.Q. A small container with KOH was placed on a shelf in the vessel for the absorption of evolved CO_2. The shelf was composed of stiff plastic and was fastened to the sides of the vessel by the pressure exerted by small pieces of rubber tubing attached to the shelf. The shape of the shelf did not interfere with maximum CO_2 diffusion from the soil into the KOH. The surface area of the KOH was adequate and filter paper wicks did not increase the rate of CO_2 absorption. No creep of the alkali was observed, thus eliminating the need for coating the rim of the container with grease. The vessels were autoclaved prior to use and plugged with cotton until they were attached to the manometers. Additions of glucose, nitrogen, phosphorus, and sulphur solutions, plus sufficient water to bring the soils to field capacity, were made to the air-dried soil (Ottawa loamy fine sand) directly in the vessels. The soils and solutions were thoroughly mixed and the vessels placed at 3° C for 24 hours in order to insure even distribution of the solutions throughout the soil. The volume of the treated soils, KOH container, and supporting shelf was determined by displacement in water (28). This method of measuring the volume of soil was most effective, since the specific gravity of the soil by

[3]American Instrument Company, Silver Springs, Maryland. Catalog No. 5-210.

this method was 2.67 g/cc and that determined in a pycnometer was 2.68 g/cc. After equilibration, the vessels were attached to the manometers, placed in a water bath, and the O_2 uptake and CO_2 evolution determined by conventional "direct" method techniques (27). The flasks were incubated at 25° C under static conditions, since gas exchange between a porous material and the gas phase is not increased by agitation as it is with a liquid system (6, 8, 19). Agitation would result in the continual rupture of mycelial strands and in the transport of spores and cells closer or further away from areas of substrate concentration, thereby upsetting the steady state that is assumed in soil incubation studies. The manometers were read for 4 to 8 hours/day at hourly intervals, except during the periods of active microbial metabolism when they were read more often. After each reading, the stopcocks on the manometers were opened in order to replenish the O_2 in the flasks. An average flask with a total volume of approximately 95 ml and filled with 25 g of soil contained approximately 15 ml of O_2. During the period of maximum microbial activity only 2 to 8 ml O_2/hour was consumed, indicating that O_2 was not deficient. After the final reading of the day, the KOH was removed from the vessels in which O_2 uptake was measured, and CO_2 collectors

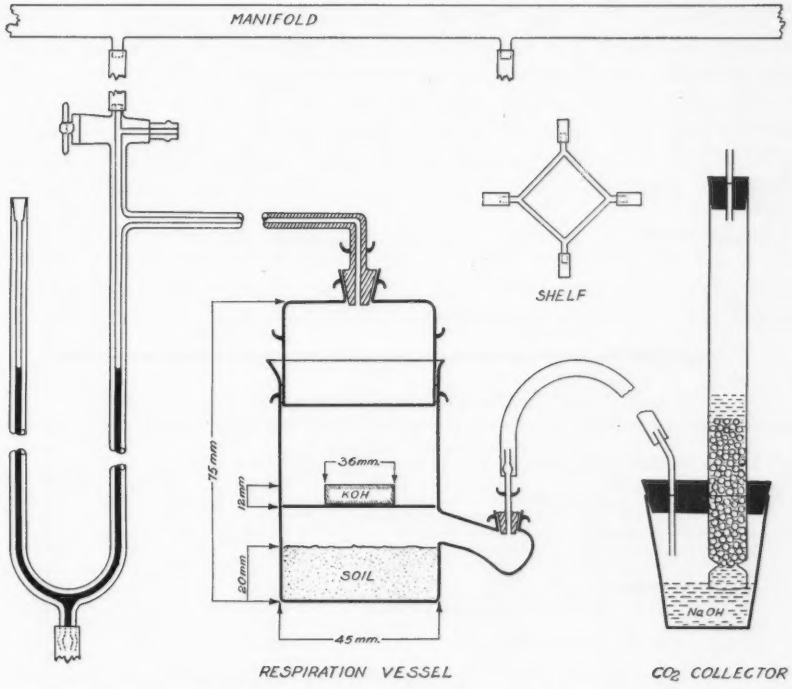

FIG. 1. Respirometer used to measure the R.Q. of soils. Shelf supports KOH container within vessel.

(25) were connected to all vessels via hollow-bore ground-glass stoppers inserted in the side arms. The manometers were attached to a manifold and the soils were continuously aerated with a stream of CO_2-free water-saturated air which flushed all evolved CO_2 into the CO_2 collectors. Prior to attaching the manometers to the manifold, the manometer fluid was adjusted to approximately the 250-mm mark which prevented the fluid from being forced out of the manometer. A diagram of the modified respirometer and the CO_2 collector is presented in Fig. 1.

Results

Preliminary experiments were conducted to test the effectiveness of the large respirometer vessels. CO_2 evolution and O_2 uptake were determined for 8 hours per day and the manometer stopcocks were left open after the final daily readings. The variation between replicates was decreased considerably by the use of the larger samples of soils as compared to the variation observed with 4 to 8 g of soil in 15-ml flasks. The vessels which contained KOH were weighed daily and the loss of water calculated by difference in weight, taking into consideration the amount of glucose lost as CO_2. This experiment was repeated several times with various levels of glucose, both in the presence and in the absence of sulphur. Sulphur was required for maximum microbial activity and provided a means of regulating the rate of respiration of this soil (24). The average of several experiments is presented in Fig. 2. Water was lost from all samples, except those treated with 2% glucose minus sulphur, and the loss increased as the level of added glucose and rate of respiration increased. Why the one treatment gained rather than lost water is not known. When the soils were aerated with a water-saturated air stream during the

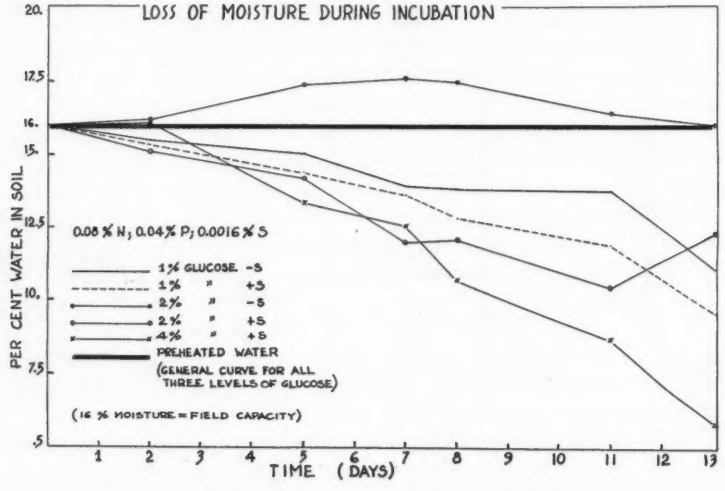

FIG. 2. Change in soil moisture during incubation.

intervals that R.Q. determinations were not conducted, i.e., between the 8-hour reading periods, there was a decrease in the amount of moisture lost. The loss was further decreased when the CO_2 collectors were attached, presumably as the result of the resistance of the hydraulic head in the CO_2 collector which slowed the flow of air over the soils and allowed equilibration between the water-saturated air stream and the soil. These modifications, however, did not entirely prevent the loss of moisture. The soils were incubated in a water bath at 25° C, but the scrubber system for the air stream was maintained at room temperature, approximately 22 to 24° C. When the water scrubber was heated to 26 to 27° C on a hot plate, the soils were maintained at the field capacity moisture content (Fig. 2). The higher temperature of the water increased the moisture content of the air stream which replaced the water removed by the KOH during the time that O_2 uptake was measured.

A possible source of error in the determination of the R.Q. by the "direct" method is that identically treated soil samples do not respire uniformly. Since the R.Q. is measured with two respirometers, differences in the microbial activity and, hence, in the rate of respiration between paired samples will result in erroneous R.Q.'s. Several experiments were conducted wherein 12 respirometers of the same treatment were established: six flasks with and six flasks without alkali, and the R.Q. was calculated from all 36 possible combinations by the "direct" method. The amount of CO_2 evolved was also measured by titration of the KOH from inside the vessels, and the R.Q. was determined for each of the six replicates on a one-flask basis, i.e., the ratio of CO_2 evolution and O_2 consumption was calculated from a single respirometer. The rate of respiration of all 12 flasks varied considerably and a highly significant difference was obtained between the resultant 36 R.Q.'s (Table I). The degree of variation in the rate of respiration and in the R.Q.'s was similar when the soil and amendments were added independently to and mixed in individual flasks, or when aliquots of the same mixture were added. The R.Q.'s determined by the one-flask method did not differ greatly, even though the rate of respiration varied significantly between flasks (Table II). The results

TABLE I

Respiratory quotients determined by the two-flask "direct" method*

μl O_2/100 g soil per hour	μl CO_2/100 g soil per hour					
	1805	2070	2245	2340	2374	2622
	Respiratory quotient					
1433	1.26	1.44	1.57	1.63	1.66	1.83
1628	1.11	1.27	1.38	1.44	1.46	1.61
1717	1.05	1.21	1.31	1.36	1.38	1.53
1754	1.03	1.18	1.28	1.33	1.35	1.49
1827	0.99	1.13	1.23	1.28	1.30	1.44
1927	0.92	1.05	1.14	1.19	1.20	1.33

Group mean $\pm$ standard error = 1.32 ± 0.034
Coefficient of variation = 15.5%

*Twelve respirometers containing the same treatment: six with and six without alkali.

TABLE II

Respiratory quotient determined by the one-flask method*

μl O_2/100 g soil per hour	μl CO_2/100 g soil per hour	R.Q.
1433	1882	1.31
1628	2210	1.36
1717	2310	1.35
1754	2323	1.32
1827	2429	1.33
1972	2693	1.37
G.M. $\pm$S.E.† = 1722 $\pm$ 74.6	2308 $\pm$ 108.6	1.34 $\pm$ 0.01
CV‡ = 10.6%	11.5%	1.8%

*Six respirometers containing the same treatment plus KOH; CO_2 evolved determined by titration.
†Group mean $\pm$ standard error.
‡Coefficient of variation.

in Tables I and II represent only one reading period of one treatment and are presented as an example of the degree of variation. Figures 3*a* and 4*a* show that the difference between the two methods of measuring CO_2 evolution markedly affect the calculation of the R.Q. over an extended incubation period. In these experiments, the KOH was titrated after 8 hours during every 24-hour reading cycle. The difference in the R.Q. as determined by the two methods was not large in the soil which received no sulphur and had a lower metabolic activity (Fig. 3*a*), but the difference was considerable, during the period of high respiration, when sulphur was added (Fig. 4*a*).

In addition to the lack of synchronization in the respiration of paired soils, there was a difference in microbial activity in the presence or absence of CO_2 in the ambient atmosphere of the vessels. During the determination of the R.Q., more CO_2 was evolved from the flasks which contained KOH than from those to which alkali had not been added (Figs. 3*b* and 4*b*). In the intervals between R.Q. determinations, less CO_2 was evolved from the flasks which contained KOH during the R.Q. determinations than from the flasks which contained no KOH (Figs. 3*c* and 4*c*). The presence of high concentrations of CO_2 in the ambient atmosphere, when the vessels were closed during the measurement of O_2 uptake, apparently inhibited maximum microbial activity, as has been reported by several investigators for a variety of biological materials (27). When CO_2 was removed from the ambient atmosphere by flushing with CO_2-free air, the respiration of the soils which respired least during the R.Q. determinations was greater, probably as the result of more substrate being available in the flasks in which microbial activity had been retarded during the 8-hour reading periods.

These experiments have been repeated a number of times with various levels of glucose. In each instance, the difference of the rate of CO_2 evolution in the presence or absence of CO_2 in the ambient atmosphere and the variation in the rate of respiration between replicate flasks was sufficiently large to affect the R.Q. Figures 3 and 4 represent an average of several experiments conducted with a 1% addition of glucose. These experiments suggest that the one-flask method is to be preferred to the two-flask "direct" method.

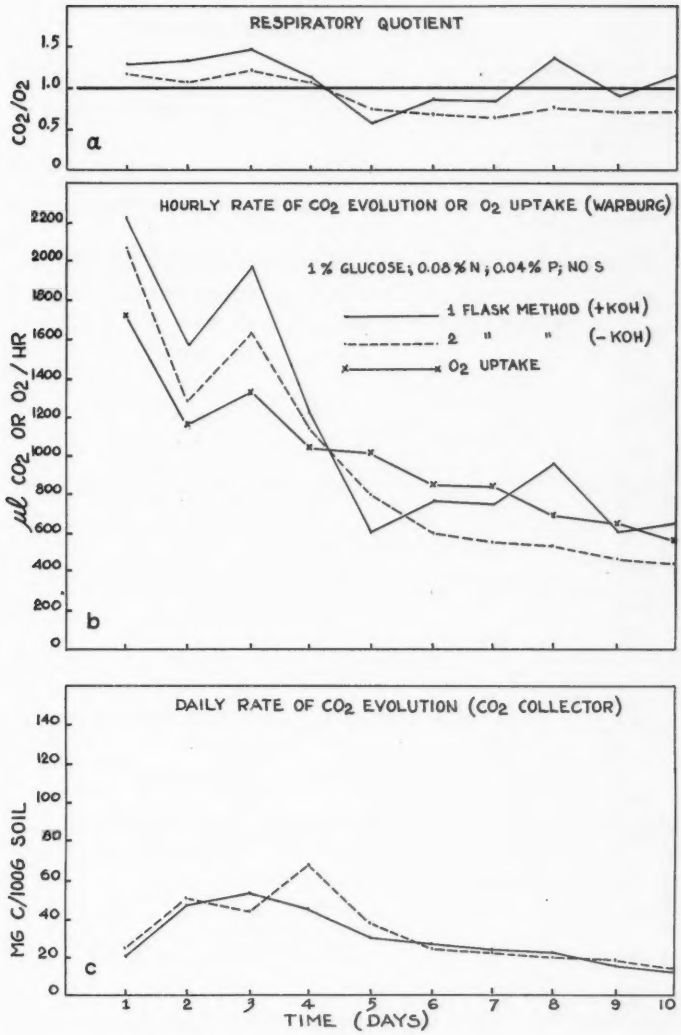

FIG. 3. Comparison of the one-flask and two-flask method of measuring the R.Q. of a soil treated with 1% glucose and no sulphur.

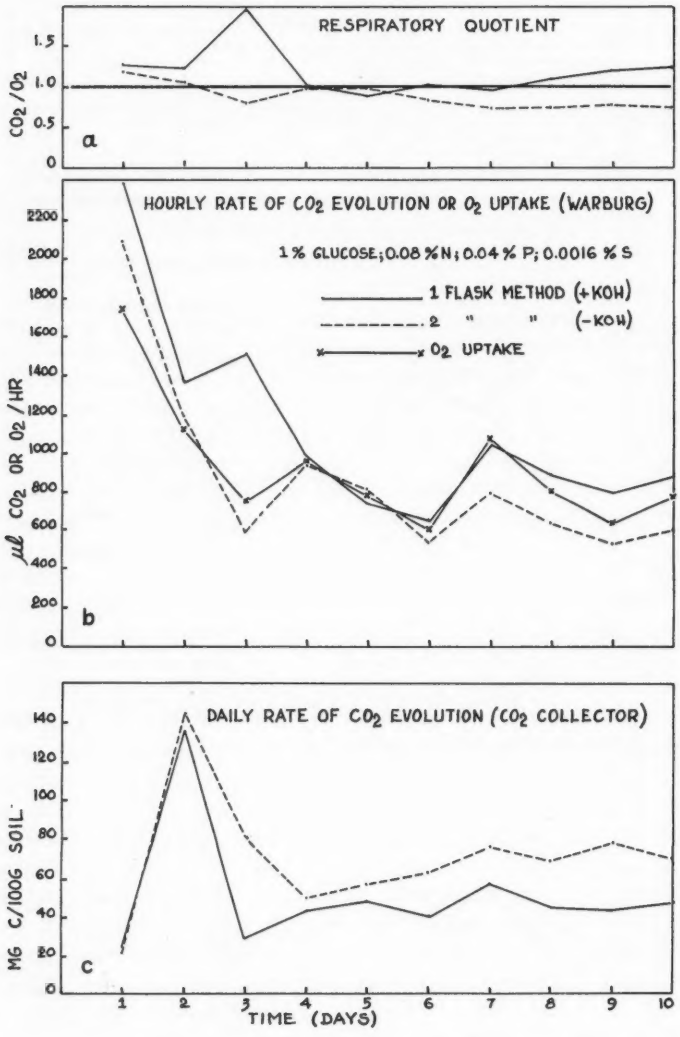

FIG. 4. Comparison of the one-flask and two-flask method of measuring the R.Q. of a soil treated with 1% glucose and sulphur.

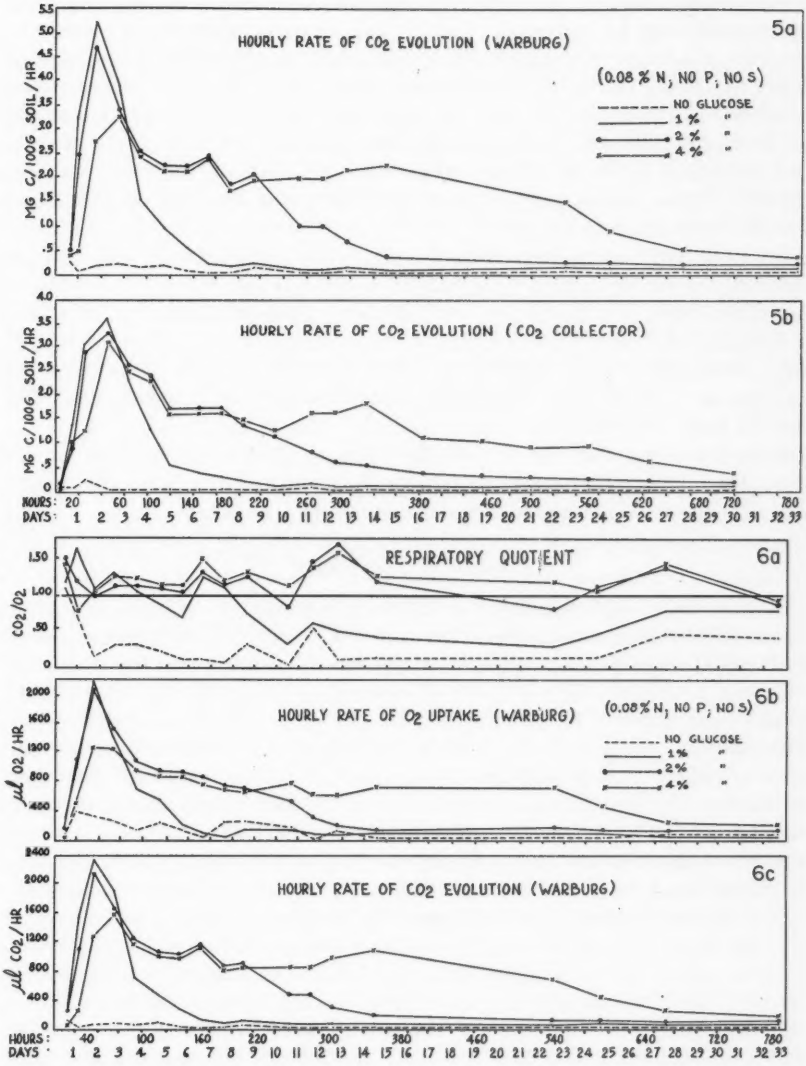
5a
HOURLY RATE OF CO2 EVOLUTION (WARBURG)
(0.08 % N, NO P, NO S)
NO GLUCOSE
1 %
2 %
4 %
MG C/100G SOIL/HR
5b
HOURLY RATE OF CO2 EVOLUTION (CO2 COLLECTOR)
MG C/100G SOIL/HR
HOURS: 20 60 100 140 180 220 260 300 380 460 500 540 620 680 720 780
DAYS: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33
6a
RESPIRATORY QUOTIENT
CO2/O2
6b
HOURLY RATE OF O2 UPTAKE (WARBURG)
(0.08 % N, NO P, NO S)
NO GLUCOSE
1 %
2 %
4 %
µl O2/HR
6c
HOURLY RATE OF CO2 EVOLUTION (WARBURG)
µl CO2/HR
HOURS: 40 100 160 220 300 380 460 540 640 720 780
DAYS: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

FIG. 5. Comparison of CO_2 evolution curves obtained by use of (*a*) respirometer (one-flask method) and (*b*) CO_2 collector.

FIG. 6. Simultaneous respiratory measurements by the one-flask method of the microbial activity of a soil treated with different levels of glucose.

The CO_2 evolution curves obtained either by titration of the KOH from the Warburg vessels or with the CO_2 collectors were similar during long-term incubations (Fig. 5). The higher rates of evolution during the early part of the incubation with the one-flask method (Fig. 5*a*) were the result of determining the R.Q. during the period of maximum respiration. The CO_2 evolution was measured for 20 hours with the CO_2 collectors and for only 4 hours with the respirometers. Since the former method encompassed a longer period of time, both high and low rates of respiration were averaged and these curves were probably more representative of the 24-hour respiration period than were the shorter measurements.

As an example of the type of data that may be obtained with the one-flask method, the hourly rates of CO_2 evolution and O_2 consumption as well as the resultant R.Q.'s of a soil treated with different levels of glucose are presented in Fig. 6. The R.Q. was measured for 4 hours daily, and the rate of CO_2 evolution during the 20-hour flushing period is shown in Fig. 5*b*. In general, the changes in the R.Q. were related to changes in the rate of respiration. The rate of both CO_2 evolution and O_2 uptake from the soil treated with 1% glucose decreased after the seventh day of incubation as did the R.Q. of this treatment. The respiration rates and the R.Q.'s of the soils which received 2 and 4% glucose remained higher throughout the incubation.

Adopted Procedure

As a result of these studies, the following procedure for the determination of the R.Q. of soils was adopted. The soils are prepared directly in the vessels and equilibrated for 24 hours at 3° C. After equilibration, the flasks are attached to the manometers. Silicone grease is a suitable lubricant for all glass joints, since it is not attacked by the soil microorganisms. The CO_2 collectors are connected to the vented side arms with hollow-bore ground-glass stoppers. The manometers are connected to the manifold, the manometer solution is adjusted to approximately the 250-mm mark, and the stopcocks are turned to engage the manifold. When the R.Q. is to be determined, the CO_2 collectors are removed and the respirometer units are transferred from the water bath to a stand. The alkali is washed from the towers of the CO_2 collectors into the tumblers with CO_2-free water, $BaCl_2$ is added to precipitate the CO_2 as $BaCO_3$, and the CO_2 is determined titrametrically with HCl on an automatic titrator (25). The bottom sections of the vessels are removed, small containers accurately filled with 2 ml of *N* KOH are placed on top of the KOH supports, and the two sections of the vessels are greased and rejoined. The hollow-bore ground-glass stoppers are replaced with solid stoppers, the units are returned to the water bath, and the flasks are allowed to equilibrate for 30 minutes. This length of equilibration was found to be adequate in most cases. Oxygen uptake is then determined by the conventional manometric procedure (27). The manometers are read hourly or over shorter intervals, depending on the microbial activity. The stopcocks are opened after each reading in order to replenish the air within the vessels and to remove any

gases which may have been produced. After 4 or 8 hours, the respirometer units are removed from the water bath, the vessels are opened, and the KOH containers are carefully removed. The KOH is transferred rapidly and quantitatively into a tumbler containing CO_2-free distilled water and $BaCl_2$, and the excess KOH is titrated with HCl on an automatic titrator (25). The total amount of CO_2 collected is corrected for the time required to read the manometers and for the original equilibration period, in order that only the CO_2 evolved during the time that O_2 uptake is actually measured is used in the calculation of the R.Q. Thermobarometers and CO_2 controls are measured concurrently with the treatments. The CO_2 collectors and the manifold are attached to the respirometer units and the soils incubated overnight in the water bath. In these experiments the soils were first incubated with the CO_2 collectors, since microbial activity during the first 20 hours of incubation was negligible and it was not deemed necessary to determine the R.Q. during this time. With soils of higher microbial activity, the R.Q.'s have been determined prior to attaching the CO_2 collectors.

Discussion

There are several advantages with the one-flask method of determining the R.Q. of soils. Aside from its simplicity, the method largely eliminates the error introduced by variation in respiration between the paired flasks which are required in the conventional two-flask "direct" method. The lack of synchronization in the rate of respiration of identically treated soils is commonly experienced in studies of soil respiration. The usual procedure of averaging the rate of respiration of several replicates is probably valid when the respiratory potential of a soil is being studied, since a sufficient number of replications is usually employed. Considerable error in the R.Q. is introduced, however, when the ratio is calculated between two replicates, the respiration of which is out of phase. The number of replicates required to minimize this error would make the method unwieldy when large numbers of determinations are to be made. The conventional Warburg water bath accommodates only 18 respiratory units, two of which are used as thermobarometers and at least two as CO_2 controls; the number of treatments that can be tested with sufficient replication during each incubation experiment is, therefore, limited to one or two. Variation in respiration will occur between replicate flasks but these will not be reflected in the R.Q., since both O_2 uptake and CO_2 evolution are measured on the same sample. The variation in the R.Q. of replicate flasks can be statistically averaged as are independent measurements of CO_2 evolution or O_2 consumption.

The moisture content of the soil can be maintained during extended periods of incubation with the one-flask method. Control of moisture is not achieved in the electrolytic or in the modified Warburg procedures, even though few reports have made reference to the variation in moisture during incubation. McGarity *et al.* (17) reported that the moisture contents after 7 and 14 days of incubation were essentially the same for a variety of treatments; however,

they were 4 to 6% lower than the initial soil moisture content. These investigators apparently did not recognize this decrease in moisture, since they make the statement that "The final moisture and O_2 content of the soil samples and vessel atmosphere respectively indicate clearly the value of the apparatus in maintaining control of these factors". Variation in moisture influences markedly the microbial activity of soils, especially of sandy soils as used in this study. Control of soil moisture during R.Q. determinations may be achieved with the continuous percolating system (11); however, the continuous disruption of soil structure, breakage of mycelial strands, and dispersion of cells and substrates throughout the soil restricts this procedure in its applicability to the study of soils in relation to their microbial activity under field conditions.

Another advantage of the one-flask method is that interference of gases other than O_2 and CO_2 is minimized. The presence of other gases will affect the measurement of O_2 uptake but not of CO_2 production, since evolved CO_2 is absorbed and measured by titration rather than by changes in pressure. Even though several investigators (17, 25) have suggested that gases other than CO_2 are probably not evolved from soil, anaerobic conditions exist and volatile gases are produced when high levels of fermentable sugars are added to the soil used in this study.

Non-biological production of CO_2 by chemical decarboxylation (4), by heat-stable enzymes (4), or by the action of organic acids formed during metabolism or added chemicals on free soil carbonates (6) may result in erroneous interpretation of the microbial activity of a soil if activity is based solely on CO_2 evolution curves. Oxygen consumption may also be non-biological, especially in heated soils as a result of reoxidation of groups which have been reduced during heating (4). The probability of non-biological CO_2 evolution or O_2 consumption indicates that periodic measurements of the R.Q. should be made in order to verify that either O_2 uptake or CO_2 evolution is a valid measure of the respiratory capacity of a specific soil.

The rate of CO_2 evolution or O_2 uptake has been commonly used as a criterion of the microbial activity of soils and has contributed greatly to the study of soil metabolism. These measurements should be evaluated periodically in order to determine that they are representative of the microbial activity of the soil being studied. Under aerobic conditions, the theoretical R.Q. of glucose decomposition should be 1. The experiments presented in this communication were conducted under apparent aerobic conditions, but the R.Q. deviated considerably from 1 throughout the entire incubation. The soil had a low organic matter content, 0.85%, and the major part of the evolved CO_2 was undoubtedly derived from decomposition of the glucose. During the early stage of the incubation, when the R.Q.'s were greater than 1, some anaerobic breakdown of glucose apparently occurred. In the latter parts of the incubation, the microbial activity decreased as a result of the complete utilization of the glucose (24) and the O_2 requirement was fulfilled by normal gas exchange. In these studies, as well as in experiments with the same soil continuously

aerated with a stream of CO_2-free air (24), anaerobiosis was apparent even though sufficient O_2 presumably was present. This suggests that the aeration status of the soil as a whole is not as important as the gas relationships in the microenvironment around the soil particles where the major part of the microbial activity takes place. The results of this study are contrary to those reported by Smith and Brown (21), who suggested that O_2 consumption or CO_2 evolution of soils may be used interchangeably since the ratio of the rate of O_2 uptake and CO_2 evolution was 1. Anaerobic activity in well-aerated soils has also been shown to occur in "aerobic denitrification" (2, 3, 12, 18, 30).

The R.Q. may provide an indication of the types of metabolic intermediates being utilized at a specific time and/or the type of population utilizing these materials. Previous experiments have shown that free glucose disappeared 3 to 4 days after addition to the soil, but only approximately 30% of the added glucose was recovered as CO_2 during this period, indicating that approximately 70% of the glucose was present in either cellular form or as intermediates between glucose and CO_2. These intermediates undoubtedly varied in their C/O ratios (23) and their decomposition was reflected in the R.Q. curves. Fluctuations in the R.Q. have also been correlated with changes in microbial numbers and types as well as with pH changes. These results will be presented in subsequent papers.

The use of CO_2 collectors in conjunction with the one-flask method makes it possible to relate changes in the R.Q. to changes in microbial activity as indicated by either CO_2 evolution or O_2 uptake. A combination of these parameters should better elucidate changes in the type of substrate being decomposed and the type of microbial population involved in the decomposition process. The apparatus required for the one-flask method is easily obtained and, with the exception of the large vessels, is available in most laboratories. The variability that is commonly observed with small volumes of soil is greatly reduced by the use of the larger vessels. If a water bath is not available, the respirometers may be incubated in a room wherein the temperature is fairly constant, since a change of 10 Centigrade degrees, in the range of 20 to 30° C, results in only a 3.6% error with the larger vessels. The composition of the ambient atmosphere in the vessels can be easily regulated by means of the manifold. Tracer studies utilizing C^{14}-enriched substrates can be conducted, since all evolved CO_2 is recovered in the alkali. The technique is easy to use and may be made completely automatic by inserting a conductivity cell into the KOH container and measuring the rate of CO_2 evolution by changes in electrical conductivity (31). The technique has been very adaptable to our experiments and publications now in preparation will further illustrate its use.

Acknowledgments

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STUDIES ON COMPLEMENT FIXING ANTIGENS OF LEPTOSPIRAE[1]

N. A. Labzoffsky and A. E. Kelen

Abstract

Methods of preparation of "whole" and type-specific antigens from leptospiral cultures for use in the complement fixation test are outlined. Leptospiral cultures grown in Korthof's medium were treated with pyridine and after appearance of a copious precipitate were centrifuged. The supernatants were dialyzed and after concentration by pervaporation were used as "whole" antigens. Type-specific antigens were prepared from acetone precipitates of filtrates of "whole" antigens. Considerable cross reaction was observed with "whole" antigens prepared from *L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae* and their respective rabbit hyperimmune sera, although titers with homologous sera were invariably higher. These cross reactions, however, were not as pronounced with sera from cattle naturally infected with *L. pomona*, where approximately 43% of the sera reacted with homologous antigen only. No reactions were observed between the type-specific antigen and any of the heterologous sera employed. It has also been shown that with experimental sera type-specific antibodies appeared earlier and persisted longer than group-specific antibodies. In view of the above observations the use of polyvalent antigen for routine screening of leptospiral antisera is advocated. Type-specific antigens are recommended for use in more precise diagnosis.

Introduction

In the sero-diagnosis of leptospirosis a number of procedures have been employed including agglutination, agglutination–lysis, and complement fixation. Agglutination or agglutination–lysis has been more commonly employed than complement fixation, though the latter is equally sensitive and specific (3) and has the advantage of not requiring the continuous maintenance of live cultures. Failure of general acceptance of complement fixation is probably due to lack of a simple and reliable procedure for the preparation of satisfactory antigens.

The early literature on the preparation and evaluation of complement fixing antigens was reviewed by Borgen (1). More recent reports deal with preparation of antigens from sonically disrupted leptospirae (2, 6), from leptospirae lysed with sodium desoxycholate (5, 8), from supernatant culture medium (2, 5), of from ethanol extracts of leptospiral cells. Two practical procedures were outlined by Terzin (9), one consisting of boiling whole culture and using it as an antigen and the other precipitating boiled culture with acetone and employing redissolved precipitate as an antigen.

In our hands Terzin's antigens have been more satisfactory than any of the others, the boiled antigen having the advantage of sensitivity and the acetone-precipitated antigen the type-specificity. The boiled antigen, however, was of low titer and contained an excessive amount of extraneous material and the acetone-precipitated antigen failed to detect small amounts of antibody.

[1]Manuscript received March 4, 1960.
Contribution from the Virus Section, Central Laboratory, Ontario Department of Health, 360 Christie Street, Toronto, Ontario. This paper was presented in part at the 27th Annual Christmas Meeting of the Laboratory Section, Canadian Public Health Association, Toronto, December 1959.

In studying the complement fixation reaction for leptospirosis, our objectives were twofold: first, for screening sera for the presence of leptospiral group antibody to develop a simple, reproducible procedure which could be applied by routine diagnostic laboratories and second, for more precise diagnosis the development of type-specific antigens.

Material and Methods

Cultures

L. icterohaemorrhagiae, *L. canicola*, and *L. pomona* were the type strains employed in this study.* The cultures were grown for 3–4 weeks at room temperature in 250-ml flasks containing 100 ml of Korthof's medium. Prior to use the cultures were examined microscopically to determine the degree of growth, and bacteriological sterility was ascertained by plating each culture on a blood agar plate.

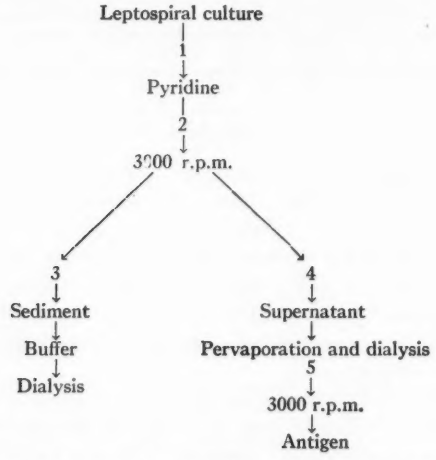

FIG. 1. Diagram of the basic procedures employed in the preparation of pyridine-extracted "whole" antigen.

Preparation of Antigens

(a) "Whole" Antigens

The procedure of preparation is outlined diagrammatically in Fig. 1. All steps were carried out at room temperature and under sterile conditions.

Step 1.—Pyridine was added to the whole culture of leptospirae. At first the culture was lysed with the production of clear fluid, but with further addition of pyridine the fluid became milky and a visible precipitate was formed. This usually occurred when the proportion of pyridine to culture was about 4:1. At this stage the materials were left in contact for 1 hour with periodic shaking.

*The *Leptospira* strains were obtained through the courtesy of the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.

Step 2.—The suspension was then centrifuged at 3000 r.p.m. for 20 minutes or, if the volume was large, it was filtered through Whatman No. 12 filter paper.

Step 3.—The sediment was redissolved in phosphate buffer 0.005 *M*, pH 7.2, and dialyzed against the same buffer to free it of traces of pyridine.

Step 4.—The supernatant from step 2 was transferred to a dialyzing bag which was half submerged in a container of running tap water placed in front of an electric fan. Pervaporation and dialysis continued until the material was concentrated to approximately tenth volume. During dialysis and pervaporation the dialyzing bag was periodically squeezed to wash off any material that might have adhered to the walls of the dialyzing bag.

Step 5.—The dialyzed material was collected, centrifuged at 3000 r.p.m. for 20 minutes, and the supernatant thus obtained was used as the antigen.

(b) Specific Antigens

Pyridine-extracted ("whole") antigens were centrifuged at 40,000 r.p.m. for 90 minutes. Supernatants so obtained were still opalescent and were filtered through ultrafine fritted glass filters. The filtrates were precipitated with 75% of their volume of acetone at room temperature. After centrifugation the sediments were dried in front of an electric fan and redissolved in distilled water to approximately 1/10 of the original volume.

Antisera

Rabbit and bovine sera were employed in this study. Immune sera, used in tests for sensitivity of the antigens, were obtained by bleeding at 2-day intervals rabbits which had received a single intravenous injection of live leptospirae. Hyperimmune sera, used in most of the tests, were prepared in rabbits by series of intravenous injections with live leptospirae.

Bovine sera were obtained through the courtesy of Dr. N. A. Fish, Ontario Veterinary College, from cattle naturally infected with *L. pomona*. *L. ballum* and *L. grippo-typhosa* antisera of guinea-pig origin were kindly supplied by Dr. R. D. Stuart, University of Alberta.

Complement Fixation Test

Details of the technique of the test were reported previously (4). Optimum dilutions of antigens and sera for the various tests were determined by grid titration. Preliminary incubation was at 37° C for 75 minutes. After addition of the haemolytic system the tests were read when all the negative controls were completely haemolyzed. All the usual controls were included in each test, but are omitted from the tables for the sake of brevity.

Results

The antigens obtained by this method were found to be of reasonably high titer. The titer of course depended directly on the degree of concentration during pervaporation. A typical example is given in Table I. All antigens prepared (22 batches) were uniformly free of anticomplementary property even in the lowest dilutions. None of the antigens prepared titered below 1:128

TABLE I

Titration of the extract ("whole" antigen) and sediment after pyridine treatment of *L. canicola*

		1 in:						
		4	8	16	32	64	128	256
Extract ("whole" antigen)	T*	++++	++++	++++	++++	++++	++++	++++
	C	−	−	−	−	−	−	−
Sediment	T	−	−	−	−	−	−	−
	C	−						

*T, test; C, antigen control. Rabbit immune serum 1:50.

and most were higher than 1:256. Copious sediments obtained (step 3, Fig. 1) during the preparation of the antigens invariably were found to be not antigenic. The antigens proved to be soluble in the true sense since they were not sedimented at 40,000 r.p.m. and were filterable through ultrafine fritted glass filter. The antigens have displayed a high degree of stability, withstanding boiling for 10 minutes, or storage for 4 months at 4° C, or for 1 year at −20° C, without significant loss of titer.

As may be seen from Table II the antigens were found to be group rather than type specific, although titers of homologous hyperimmune sera were somewhat higher. Cross reactions with *L. icterohaemorrhagiae* antiserum were less pronounced than with the other sera.

Sensitivity of the Antigens

The sensitivity of the antigens prepared by the pyridine extraction method was evaluated by comparing them with Terzin's acetone-precipitated and boiled antigens (9), which, as was mentioned earlier, we consider to be superior to antigens prepared by any other method.

In testing pyridine-extracted antigens for sensitivity we were particularly interested in determining how early in the course of experimental infection the antibodies could be detected by complement fixation. For this purpose trial bleedings from a *canicola*-infected rabbit taken at 2-day intervals were tested against the three antigens, pyridine-extracted, Terzin's acetone-

TABLE II

Cross fixation reactions between *L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae*

	Serum, 1:						
	8	16	32	64	128	256	Antigen
L. pomona rabbit No. 11	++++	++++	++++	++++	++++	+++	1*
	++++	++++	++++	+++	++	−	2
	++++	++++	++++	+++	++	−	3
L. canicola rabbit No. 12	++++	++++	++++	+++	+	−	1
	++++	++++	++++	++++	++++	+++	2
	++++	++++	++++	++++	+++	++	3
L. icterohaemorrhagiae rabbit No. 7	++++	+++	++	+	−	−	1
	+++	++	+	−	−	−	2
	++++	++++	++++	++++	++++	+++	3

*1, *L. pomona* "whole" pyridine-extracted antigen;
2, *L. canicola* "whole" pyridine-extracted antigen;
3, *L. icterohaemorrhagiae* "whole" pyridine-extracted antigen.

TABLE III

Relative sensitivity of Terzin's acetone-precipitated, Terzin's boiled, and "whole" pyridine-extracted antigens. *L. canicola* infected rabbit No. 12

	Serum, 1:						
	8	16	32	64	128	256	Antigen
2 days after infection	−	−	−	−			1*
	−	−	−	−			2
	−	−	−	−			3
4 days after infection	−	−	−	−			1
	+++	++	−	−			2
	++++	+++	++	−			3
7 days after infection	++++	++++	++++	+++	−	−	1
	++++	++++	++++	+++	++	+	2
	++++	++++	++++	++++	++++	+++	3
9 days after infection	++++	++++	++++	+++	++	−	1
	++++	++++	++++	++++	+++	++	2
	++++	++++	++++	++++	++++	+++	3
11 days after infection	++++	++++	++++	+++	++	−	1
	++++	++++	++++	++++	++++	+++	2
	++++	++++	++++	++++	++++	+++	3

*1, Terzin's acetone-precipitated *L. canicola* antigen;
2, Terzin's boiled *L. canicola* antigen;
3, "whole" pyridine-extracted *L. canicola* antigen.

precipitated, and Terzin's boiled, prepared from *L. canicola*. The results of one of such tests are recorded in Table III. As is shown by this table antibodies can be detected on the fourth day by Terzin's boiled antigen and by pyridine-extracted antigen, but not until the seventh day, by Terzin's acetone-precipitated antigen. Pyridine-extracted antigen appears to be somewhat more sensitive than the others, though the difference between pyridine-extracted and the boiled antigens was not significant after the seventh day.

Time of Appearance and Persistence of Group- and Type-specific Antibodies

In order to determine which of the two antibodies—type or group specific—appears first, the same trial bleedings used in the previous experiment (Table III) were titrated against the three pyridine-extracted antigens prepared from *L. pomona*, *L. icterohaemorrhagiae*, and *L. canicola*. The results which are tabulated in Table IV show that the type-specific antibody was detectable

TABLE V

Time of appearance of type-specific and group-specific antibodies in the sera of *L. canicola* infected rabbit No. 12

	Serum, 1:						
	8	16	32	64	128	256	Antigen
4 days after infection	−	−	−	−			*L. pomona*
	−	−	−	−			*L. icterohaemorrhagiae*
	++++	+++	+	−			*L. canicola*
7 days after infection	++++	+++	+	−	−		*L. pomona*
	++++	++++	++++	++	−		*L. icterohaemorrhagiae*
	++++	++++	++++	++++	++++	++	*L. canicola*
9 days after infection	++++	++++	+++	++	+	−	*L. pomona*
	++++	++++	++++	+++	+	−	*L. icterohaemorrhagiae*
	++++	++++	++++	++++	++++	+++	*L. canicola*
11 days after infection	++++	++++	++++	+++	+	−	*L. pomona*
	++++	++++	++++	++++	+++	−	*L. icterohaemorrhagiae*
	++++	++++	++++	++++	++++	+++	*L. canicola*

4 days after infection, whereas the group-specific antibody did not appear until the seventh day. The maximum titer in all cases was reached by the 11th day.

To establish the time of disappearance of antibodies, weekly bleedings from another rabbit previously infected with *L. canicola* were tested against the same three antigens. Up to the seventh week after infection the antibody titers remained at a constant level. From the seventh week on there was a gradual decline in the titers. The results of these observations are recorded in Table V, which shows that the antibody for *L. pomona* disappeared by the end of the eighth week and that for *L. icterohaemorrhagiae* by the end of the 16th week. The type-specific antibody for *L. canicola*, on the other hand, was still detectable 24 weeks after infection. The results of these two experiments seem to indicate that type-specific antibodies appear earlier in the course of infection than heterologous antibodies and persist longer.

TABLE V

Time of disappearance of type- and group-specific antibodies in the sera of *L. canicola* infected rabbit No. 16

	Serum, 1:					
	8	16	32	64	128	Antigen
7 weeks after infection	++++	++	+	−	−	*L. pomona*
	++++	++++	+++	−	−	*L. icterohaemorrhagiae*
	++++	++++	++++	++++	+++	*L. canicola*
8 weeks after infection	+++	−	−	−		*L. pomona*
	++++	+++	−	−		*L. icterohaemorrhagiae*
	++++	++++	++++	+++	−	*L. canicola*
12 weeks after infection	−	−	−	−		*L. pomona*
	++++	++	−	−		*L. icterohaemorrhagiae*
	++++	++++	+++	+	−	*L. canicola*
16 weeks after infection	−	−	−	−		*L. pomona*
	+++	−	−	−		*L. icterohaemorrhagiae*
	++++	++++	++	−		*L. canicola*
20 weeks after infection	−	−	−			*L. pomona*
	−	−	−			*L. icterohaemorrhagiae*
	++++	+++	+	−		*L. canicola*
24 weeks after infection	−	−	−			*L. pomona*
	−	−	−			*L. icterohaemorrhagiae*
	+++	−	−	−		*L. canicola*
28 weeks after infection	−	−	−			*L. pomona*
	−	−	−			*L. icterohaemorrhagiae*
	−	−	−	−		*L. canicola*

Complement Fixation Reactions with Sera from Cattle Naturally Infected with L. pomona

To compare the degree of reaction with homologous and heterologous leptospiral antigens, 28 sera from cattle naturally infected with *L. pomona* were titrated against *L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae* antigens. The results obtained fell into three groups, a typical example of each being given in Table VI. Fourteen out of 28 sera reacted with all three antigens, the titers in all cases being highest with homologous antigen. Two reacted with *L. pomona* and *L. canicola* and 12 with L. *pomona* only. The sera may have come from cattle in different stages of infection, since the results resemble

TABLE VI

Complement fixation reactions with sera from cattle naturally infected with *L. pomona*, using *L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae* "whole" antigens

	Serum, 1:					
	8	16	32	64	128	Antigen
I	++++	++++	++++	++++	++++	*L. pomona*
	++++	++++	++++	+++	−	*L. canicola*
	++++	++++	+++	++	−	*L. icterohaemorrhagiae*
II	++++	++++	++++	++++	+++	*L. pomona*
	++++	+++	++	−	−	*L. canicola*
	−	−	−	−	−	*L. icterohaemorrhagiae*
III	++++	++++	++++	++++	+++	*L. pomona*
	−	−	−	−	−	*L. canicola*
	−	−	−	−	−	*L. icterohaemorrhagiae*

those found with experimental antisera (Tables IV and V) in which type-specific antibody appeared earlier and persisted longer than group-specific antibody.

Relative Purity of Pyridine-extracted Antigens

The pyridine-extracted antigens were further compared for relative purity with Terzin's boiled and acetone-precipitated antigens. This was determined with the aid of electrophoresis. The results of this comparison are presented in Fig. 2, which shows that Terzin's boiled and acetone-precipitated *L. canicola*

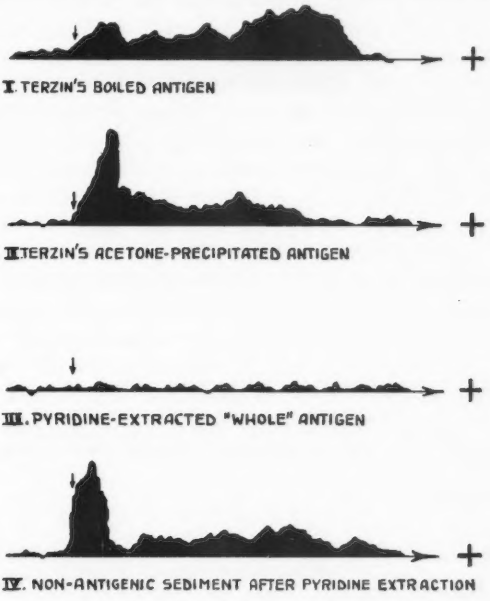
I. TERZIN'S BOILED ANTIGEN
II. TERZIN'S ACETONE-PRECIPITATED ANTIGEN
III. PYRIDINE-EXTRACTED "WHOLE" ANTIGEN
IV. NON-ANTIGENIC SEDIMENT AFTER PYRIDINE EXTRACTION

FIG. 2. Relative purity of various antigens as determined by paper electrophoresis.

antigens contained considerable protein material, the amount of which practically equalled that of the non-antigenic precipitate obtained during pyridine extraction. The pattern of fractionation of Terzin's boiled and acetone-precipitated antigens and pyridine insoluble, non-antigenic precipitate closely resembles that of serum. The pyridine sediment in particular contains almost the entire range of serum proteins. This may be due to the presence of serum in the culture medium. Pyridine-extracted antigen, on the other hand, contains no demonstrable protein. Carbohydrates, also, were not detectable in pyridine-extracted antigens. Pilot tests indicated that the pyridine-extracted antigen may be of the nature of a nucleic acid complex.

Type-specific Acetone-extracted Antigens

A number of batches of type-specific antigens prepared from *L. pomona*, *L. canicola*, and *L. icterohaemorrhagiae* indicated that the procedure employed for the preparation of such antigens was reproducible. Yield of the antigens in comparison with the amount of starting material was rather small and to obtain reasonably high titers required five- to ten-fold concentration. The soluble antigens so prepared were found to be free of anticomplementary properties even when undiluted. Boiling these antigens for 10 minutes did not appreciably reduce their titers. They were stable at 4° C for at least 2 months. The specificity of the antigens was determined by cross fixation against heterologous antisera. The results are recorded in Table VII. Hyperimmune *L. pomona*, *L. icterohaemorrhagiae*, and *L. canicola* rabbit sera employed in this test had titers of 1:256 against homologous antigens. *L. ballum* and *L. grippotyphosa* antisera were of guinea-pig origin with agglutination–lysis titers of 1:10,000 and 1:300 respectively. Two units of antigen were used in each case and serum was diluted 1:8. The results show that in each case specific antigen reacted only with its homologous antiserum. For comparison pyridine-extracted "whole" antigens were also included in the test. Lack of type specificity of "whole" pyridine-extracted antigens is apparent in Table VII and is especially notable with *L. icterohaemorrhagiae* serum.

TABLE VII

Specificity of the type-specific leptospirae antigens as determined by cross fixation against heterologous antisera

	Antiserum, 1 in 8				
Antigen	*L. pomona*	*L. icterohaemorrhagiae*	*L. canicola*	*L. grippotyphosa*	*L. ballum*
Type-specific antigens					
L. pomona	++++	−	−	−	−
L. icterohaemorrhagiae	−	++++	−	−	−
L. canicola	−	−	++++	−	−
"Whole" antigens					
L. pomona	++++	++++	++++	−	+
L. icterohaemorrhagiae	++++	++++	++++	++++	+++
L. canicola	++++	++++	++++	−	++

TABLE VIII

Sensitivity of "whole" pyridine-extracted antigen, type-specific antigen, and Terzin's acetone-precipitated antigen

	Serum, 1:					
	8	16	32	64	128	Antigen
L. pomona rabbit No. 11	++++	++++	++++	++++	+++	*L. pomona* 1*
	++++	++++	++++	+++	++	2
	++++	++++	++++	++	+	3
L. canicola rabbit No. 9	++++	++++	++++	+++	++	*L. canicola* 1
	++++	++++	++++	++	+	2
	++++	++++	++	+	−	3
L. icterohaemorrhagiae rabbit No. 17	++++	++++	++++	+++	−	*L. icterohaemorrhagiae* 1
	++++	++++	+++	+	−	2
	++++	++++	++	−	−	3

*1, "Whole" pyridine-extracted antigen;
2, type-specific antigen;
3, Terzin's acetone-precipitated antigen.

In Table VIII the sensitivities of "whole" antigens, Terzin's acetone-precipitated antigens, and our type-specific antigens for homologous antibodies are compared. In each case the "whole" pyridine-extracted antigen is rather the most sensitive, but the type-specific antigens compare favorably with Terzin's.

Comments

The procedure outlined in this communication for the preparation of "whole" leptospiral antigens was found to be reliable in giving reproducible results and sufficiently simple to be practical. The antigens prepared by this method were stable and contained only a minimum of extraneous material. They were soluble in the true sense, since they were not sedimented at 40,000 r.p.m. and were filterable through ultrafine fritted glass filter.

The antigens so prepared were found to be sensitive in detecting the early appearance of antibodies. In experimental rabbit infections these antibodies showed considerable cross reaction with heterologous antigens, but the dominance and persistence of purely specific reactions in both experimentally infected rabbits and naturally infected cattle has been demonstrated. Therefore it is obvious that for routine screening of sera the use of polyvalent antigen is necessary. Antigens prepared by pyridine extraction, being of a reasonably high titer (1:256 or higher), are suitable for use in the polyvalent preparations. Because of a relatively low yield of the highly specific acetone-extracted antigens their use in the routine screening does not appear to be practical. However, they have value in precise diagnosis of the type of *leptospira* involved in a given case.

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STUDIES ON LATEX AGGLUTINATION TEST FOR LEPTOSPIROSIS[1]

A. E. Kelen and N. A. Labzoffsky

Abstract

Experiments were carried out on the applicability of the latex fixation principle for the serological diagnosis of leptospirosis. Polystyrene latex particles suspended in a buffer solution were coated with soluble antigens of *Leptospira canicola*, *L. icterohaemorrhagiae*, and *L. pomona*, respectively, and used as agglutinating antigens for testing sera of various origin. The optimal conditions for the preparation of the test antigens, as well as for the procedure employed in performing the leptospiral latex agglutination test, were determined.

The results of the leptospiral latex agglutination test were found to be quite comparable in specificity and sensitivity with those of other serological tests commonly used in laboratory practice. The advantages of the leptospiral latex agglutination test over some other serological tests are found in the simplicity and rapidity of its performance and in the use of a non-infective, stable antigen, which eliminates the necessity of maintaining live leptospiral cultures in diagnostic laboratories.

Introduction

Investigations of recent years have shown that latex particles can be successfully used in agglutination tests for various antigen–antibody systems. The latex fixation technique was developed by Singer and Plotz (8), who applied it to the serological diagnosis of rheumatoid arthritis (6). Its usefulness in diagnosing rheumatoid arthritis was confirmed later by several workers (2, 7, 9). It proved to be applicable also to the serodiagnosis of histoplasmosis (1) and some staphylococcal infections (11). Muraschi (5) described a "latex leptospiral agglutination test", using formolized leptospirae for preparation of the test antigen. Muraschi's antigen, however, does not materially differ from other antigens containing whole leptospiral organisms and used in the ordinary leptospiral agglutination test. According to Singer and Plotz (8), it seems likely that the latex fixation is essentially a precipitation reaction between soluble antigens coated on the surface of the biologically inert latex particles and the corresponding antibodies, the reaction being manifested by aggregation of the particles.

In our previous paper (4), a method was described for the preparation of soluble antigens of leptospirae by pyridine extraction. The present paper deals with the results of investigations on the applicability of the latex agglutination test in the serodiagnosis of leptospirosis, using soluble leptospiral antigens adsorbed to the surface of latex particles.

[1]Manuscript received April 8, 1960.
Contribution from the Virus Section, Central Laboratory, Ontario Department of Health, 360 Christie Street, Toronto, Ontario. This paper was presented in part at the 27th Annual Christmas Meeting of the Laboratory Section, Canadian Public Health Association, Toronto, December 1959.

Materials and Methods

Leptospiral Strains

Three strains of leptospirae, *L. canicola*, *L. icterohaemorrhagiae*, and *L. pomona*, were employed in these studies. The origin of the strains and the method of propagation have been reported elsewhere (4).

Antisera

The antileptospiral immune and hyperimmune rabbit sera, and the bovine sera, used in these experiments were the same as those employed in our previous work in connection with the complement fixation test (4). Antileptospiral human serum was from an individual naturally infected with *L. canicola*. As controls, normal rabbit, human, and bovine sera, sera from rabbits hyperimmunized against other organisms, and sera from cattle and humans suffering from other infections were also included.

Latex Suspension

Polystyrene latex particles 0.81 μ in diameter were used in the form of a thick, white, semifluid suspension, available commercially.* The commercial preparation was diluted 1:2.5 with distilled water, filtered through Whatman No. 40 filter paper, and stored at 4° C until used as latex stock suspension.

Diluent

Borate-buffered saline solution of pH 8.2 (8) served as the diluent for the preparation of both the antigen suspensions and serum dilutions.

Basic Antigens

The preparation of leptospiral "whole" antigens, which henceforth will be referred to as basic antigens, has been described elsewhere (4).

Test Antigens

The latex stock suspension was diluted with borate-buffered saline solution to make a suspension of an optical density of 0.30 as determined by the Coleman Nepho-colorimeter at a wave length of 665 mμ. The test antigens were prepared by mixing 1 part of undiluted leptospiral basic antigen with 19 parts of the final latex dilution. After overnight adsorption at room temperature, the test antigens were kept refrigerated at 4° C until used. Test antigens so prepared and stored were found to be stable for at least 9 months.

The Test

To twofold serial serum dilutions in 0.5-ml volumes, equal amounts of test antigen were added. As a control on possible autoagglutination, tubes containing 1 ml of each test antigen without serum were included with all tests. Following incubation at 37° C for 2 hours, the tubes were centrifuged at 2000 r.p.m. for 5 minutes, and the results read by the naked eye.

Reading of the results was accomplished with the aid of obliquely transmitted light against a dull black background or by the use of a suitable

*Dow Chemical Company, Midland, Michigan; or Hyland Laboratories, Los Angeles, California.

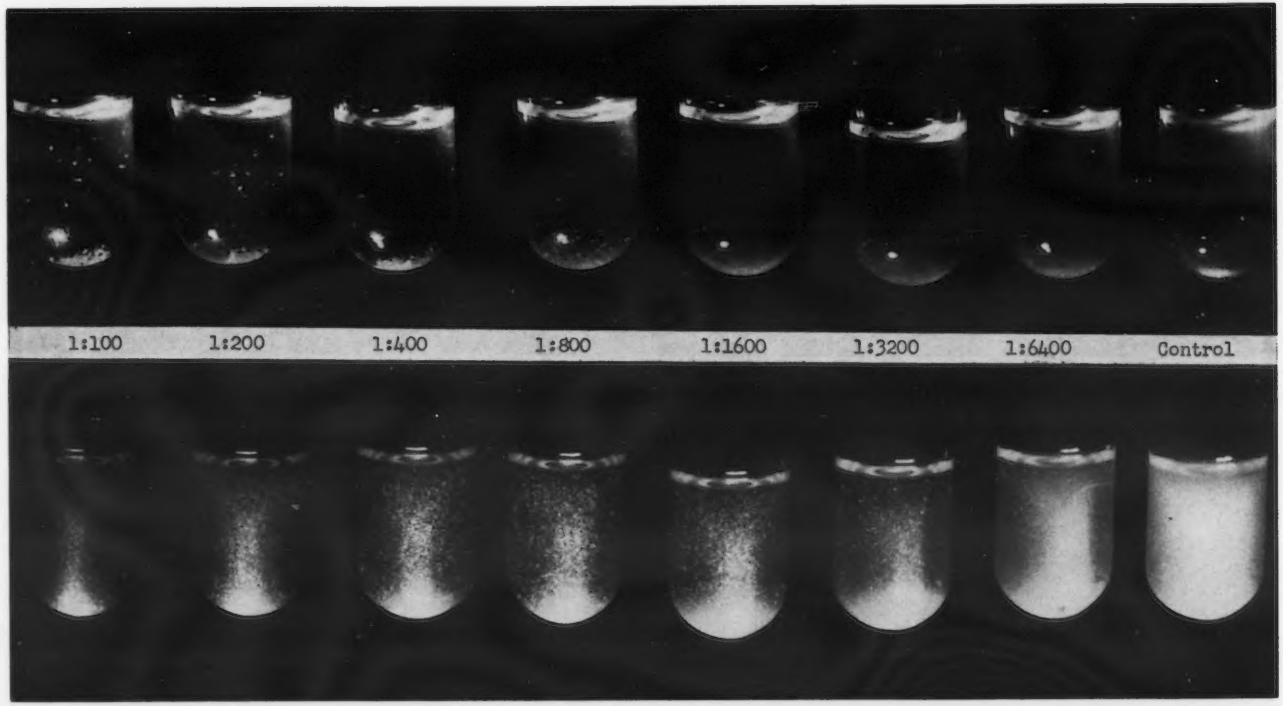

FIG. 1. Macroscopic latex agglutination test. Upper row: after centrifugation. Lower row: after gentle shaking.

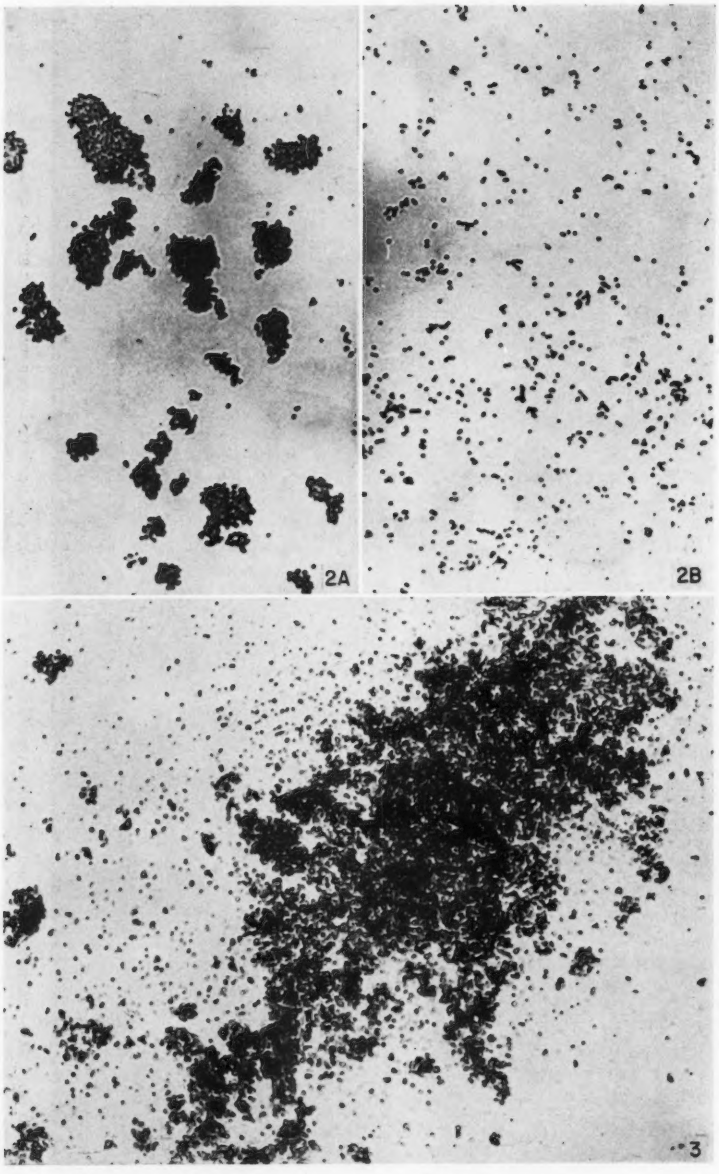

FIG. 2. Microscopic latex agglutination test. ×128
A: Aggregates of antigen-coated latex particles from positive tube.
B: Antigen-coated latex particles from control tube.
FIG. 3. Microscopic latex agglutination test. Aggregation of latex particles from positive tube. ×640

illuminator (agglutinoscope). As shown in Fig. 1, a more or less sharply circumscribed, round, compact sediment was seen at the bottom of the control and negative tubes. This sediment was, however, readily and smoothly resuspended into a homogenous milky suspension by slight tapping or rotating or shaking of the tube. In positive tubes, a well-developed aggregate or a loosely dispersed and coarsely granular sediment covered the bottom of the tubes. After the tubes were shaken, the agglutinated particles were clearly visible in the suspension. The end-titer of a serum was taken as the highest dilution which caused agglutination of 50% or more of the latex particles.

Alternatively, centrifugation may be omitted, and a drop of the suspension placed on a slide without cover slip and examined under low power magnification (100× – 200×). As seen in Fig. 2, the control and negative tubes showed the presence of evenly distributed single latex particles. In positive samples, on the other hand, smaller or larger groups of aggregated latex particles were seen, accompanied by a significant decrease in the number of single particles. When examined under higher magnification (Fig. 3), the structure of the aggregates was clearly seen to be composed of individual latex particles closely attached to one another.

Other Serological Tests

The microagglutination–lysis test was performed according to Wolff (10). The macroagglutination test was carried out in tubes, by using 1- to 2-week-old live cultures of leptospirae (3). The technique of the complement fixation test used in these studies was reported in our previous paper (4).

Development of the Procedure

Selection of Electrolytes and Basic Antigens

Of three electrolytes tested, the borate-buffered saline solution proved to be the most suitable diluent for preparation of the test antigens as well as the serum dilutions. It was tested in a pH range from 7.2 to 9.2 with intervals of pH 0.5. The optimal pH was determined as 8.2 for all three leptospiral strains.

Four varieties of leptospiral basic antigens were employed for coating latex particles: (1) Muraschi's formolized antigen, (2) Terzin's boiled antigen, (3) ultrasonorated, and (4) pyridine-treated cultures.

(1) Muraschi's antigen, as seen by dark-field examination, consisted of intact leptospirae, either single or clumped together in smaller or larger groups, dispersed among individual latex particles. Some of the latex particles appeared to be adherent to leptospiral clumps or to individual leptospirae, but most of them were floating freely in the fluid. As a result of agglutination by homologous antibodies, a sediment was formed consisting of intact leptospirae clumped together and intermingled with evenly distributed latex particles, no aggregation of the latter being detectable. Macroscopically, the sediment was of a fluffy type, similar to that observed in the ordinary leptospiral tube agglutination test. It appears, therefore, that in Muraschi's formolized antigen only whole leptospirae are responsible for the reaction, and the latex particles

TABLE I

Results of testing of different basic antigens

L. pomona basic antigens	*L. pomona* hyperimmune rabbit serum								
	1:50	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	Control
Formolized	++++	++++	++++	++++	(++++)	(+++)	(++)	±	±
Boiled	−	−	−	−	−	−	−	−	−
Ultrasonorated	++++	+++	++	+	−	−	−	−	−
Pyridinized	++++	++++	++++	++++	++++	(++++)	+++	++	−

NOTE: Parentheses indicate somewhat weaker reactions than those expressed by the number of + signs.

cannot be considered as an essential contributing factor. As seen in Table I, Muraschi's formolized antigen proved to be of a relatively high sensitivity, but showed a slight degree of spontaneous agglutination.

(2) Terzin's boiled antigen prepared by heating leptospira cultures in the water bath at 100° C for 15 minutes (4), when examined by dark-field microscopy, was seen to contain many intact leptospirae as well as leptospiral fragments scattered among single latex particles. No agglutination of latex particles took place even with the starting dilution of 1:50 of the homologous antiserum (Table I).

(3) In the ultrasonorated antigen, a few whole leptospiral organisms, more fragments, but mostly mere granules could be seen dispersed among individual latex particles. In Table I, it is shown that this antigen gave somewhat better results than the boiled, but was still of low sensitivity.

(4) When examined under the dark-field microscope, the pyridine-treated test antigen showed the presence of single latex particles only, without any leptospiral organisms, fragments, or granules. The best results were obtained with this antigen, which invariably proved to be of reasonably high sensitivity, as shown in Table I.

Determination of the Optimal Concentrations

The optimal density of the final latex suspension was determined by testing antigens prepared with borate-buffered saline solutions containing decreasing concentrations of the latex stock suspension from 1% to 0.1%. The best results were obtained with a concentration of 0.6%, which corresponded to an optical density of 0.30 at a wave length of 665 mμ as determined by the Coleman Nepho-colorimeter.

The optimal concentration of the basic antigens in the test antigens was determined by testing different batches of test antigens containing increasing amounts from 1% to 10% of the basic antigen (Table II). The optimal concentration was found to be 7%. Higher basic antigen content gave no better results, and was accompanied by some autoagglutination. All further tests were carried out with test antigens containing 5% basic antigen, which proved to be the minimal concentration that yielded a high sensitivity, and had the advantage of some economy of material.

The Influence of Time and Temperature upon Adsorption

To determine the influence of time and temperature upon the rate of adsorption, *L. canicola* basic antigen was kept in contact with latex suspension

TABLE II

Determination of the optimal basic antigen concentration

L. canicola basic antigen concentration	*L. canicola* hyperimmune rabbit serum							
	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	Control
1%	++++	++++	+++	(++)	−	−	−	−
3%	++++	++++	+++	(+++)	++	+	−	−
5%	++++	++++	++++	(++++)	+++	++	+	−
7%	++++	++++	++++	(++++)	+++	(+++)	++	−
10%	++++	++++	(++++)	+++	(+++)	(++)	+	+

NOTE: Parentheses indicate somewhat weaker reactions than those expressed by the number of + signs.

TABLE III

Influence of time and temperature upon adsorption

Adsorption	*L. canicola* hyperimmune rabbit serum								
	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600	Control
At 56° C for ½ hr	+++	+++	(+++)	(++)	−	−	−	−	−
At 56° C for 2 hr	+++	++	+	−	−	−	−	−	−
At 37° C for 2 hr	++++	++++	++++	++++	(++++)	+++	++	−	−
At 37° C overnight	++++	++++	+++	(+++)	++	+	−	−	−
At 22° C for 2 hr	++++	++++	++++	++++	(++++)	+++	++	−	−
At 22° C overnight	+++	++++	++++	++++	(++++)	+++	(+++)	(++)	−
At 4° C overnight	++++	++++	++++	(++++)	+++	++	+	−	−
At 4° C for 1 week	++++	++++	++++	++++	(++++)	+++	(++)	−	−

NOTE: Parentheses indicate somewhat weaker reactions than those expressed by the number of + signs.

TABLE IV

Influence of incubation upon the test

Incubation	*L. canicola* hyperimmune rabbit serum						
	1:800	1:1600	1:3200	1:6400	1:12,800	1:25,600	Control
At 56° C for ½ hr	+++	(++++)	+++	(+++)	(++)	−	−
At 56° C for 2 hr	(++++)	+++	(+++)	++	−	−	−
At 37° C for 2 hr	(++++)	+++	(+++)	++	(++)	−	−
At 37° C overnight	++++	++++	++++	+++	++	+	−
At 22° C for 2 hr	+++	(+++)	(++)	−	−	−	−
At 22° C overnight	(++++)	+++	++	+	±	−	−

NOTE: Parentheses indicate somewhat weaker reactions than those expressed by the number of + signs.

at 56° C, at 37° C, at room temperature, and at 4° C, respectively, for various periods of time (Table III). Adsorption at lower temperatures gave antigens of higher sensitivity, and required a longer time of contact. It is interesting to note that at higher temperatures higher sensitivity was obtained with shorter periods of contact. For routine production of test antigens, overnight adsorption at room temperature was found to be the most satisfactory procedure.

The Influence of Various Factors upon the Test

Incubation.—*L. canicola* antigen–antibody reaction was tested by employing various incubation periods at different temperatures (Table IV). Overnight incubation in the water bath at 37° C proved to be the most suitable, though

incubation at 56° C for ½ hour or at 37° C for 2 hours with subsequent overnight refrigeration also gave good results. Overnight incubation at room temperature was found to be less satisfactory. If results are required within a few hours, the tests may be incubated at 56° C for ½ hour or at 37° C for 2 hours without overnight refrigeration. Reading of such tests is, however, more difficult, and end points are not so clear-cut.

Centrifugation.—It was found that even after optimal incubation no grossly visible agglutination developed in the antigen–antibody system. Agglutinated latex particles formed clumps of only microscopic size at this stage. Sedimentation of these aggregates by gravitation proved to be a very slow process requiring several days, and resulted only in a very finely granular sediment, unsatisfactory for differentiating between positive and negative reactions by the naked eye. The application of centrifugal force proved to be effective not only in accelerating significantly the process of sedimentation, but also by forming a coherent, compact sediment in the positive tubes. This could not be resuspended into a homogenous milky suspension by shaking, but broke up into smaller or larger groups of aggregated latex particles, readily visible macroscopically in the suspension. In the negative or control tubes, the sediment formed after centrifugation could be readily resuspended. In order to determine the proper speed and time of centrifugation, the tubes were spun at 1000 to 4000 r.p.m. for 1 to 15 minutes. Using low speeds for short periods (1000 r.p.m. for 1 to 5 minutes or 2000 r.p.m. for 1 to 2 minutes) proved to be unsatisfactory, resulting in sediments which could be more or less completely resuspended even in positive tubes. The application of higher speeds (3000 to 4000 r.p.m.) produced autoagglutination, which increased with the time of centrifugation. No autoagglutination was observed by using 1000 r.p.m. for 1 to 15 minutes or 2000 r.p.m. for 1 to 10 minutes, the latter giving better results than the former. For routine performance of the test, centrifugation at 2000 r.p.m. for 5 minutes proved to be the most reliable and efficient procedure.

Inactivation of sera.—It was found that there was no significant difference in the end-titer of the sera, whether inactivated at 56° C or not. Inactivated sera, however, gave somewhat more clear-cut results.

Evaluation of the Test

Specificity of the Test

Using test antigens prepared from *L. canicola*, *L. icterohaemorrhagiae*, and *L. pomona*, respectively, latex agglutination tests (henceforth referred to as LAT) were performed on a total of 60 sera of rabbit, bovine, and human origin. Twenty of these (9 hyperimmune rabbit sera, and 1 human and 10 bovine convalescent sera) contained type-specific antibodies against one of the above leptospira strains as determined previously by the micro-agglutination–lysis test (MALT), the macro-agglutination test (MAT), and the complement fixation test (CFT). Another 20 of the sera (8 hyperimmune rabbit sera, and 6 human and 6 bovine convalescent sera) revealed the presence of

TABLE V

Specificity of latex agglutination test

Sera	Antigens		
	L. canicola	*L. icterohaemorrhagiae*	*L. pomona*
Rabbit, hyperimmune, LC-587 (*canicola*)	6400	100	400
Rabbit, hyperimmune, LI-586 (*icterohaemorrhagiae*)	100	3200	800
Rabbit, hyperimmune, LP-586 (*pomona*)	400	100	6400
Rabbit, hyperimmune, BR-583 (*brucella*)	—	—	—
Rabbit, normal, NO-945	—	—	—
Bovine, convalescent, E-2482 (leptospirosis)	100	100	1600
Bovine, convalescent, E-1507 (Q fever)	—	—	—
Bovine, normal, E-2685	—	—	—
Human, convalescent, K-1614 (leptospirosis)	3200	200	100
Human, convalescent, H-4180 (syphilis)	—	—	—
Human, normal, H-2578	—	—	—

NOTE: — = Negative in a dilution of 1:100. Titers are expressed in reciprocals.

antibodies against microorganisms other than leptospirae (such as brucellae, salmonellae, shigellae, escherichiae, rickettsiae, and spirochetes, respectively) as shown by agglutination test or CFT. The remaining 20 were normal sera obtained from healthy individuals.

In Table V, the results of the LAT performed with representative sera only are shown. Negative results were obtained in dilutions of 1:100 or higher with all normal sera, with rabbit sera prepared by hyperimmunization with microorganisms other than leptospirae (e.g. brucellae), and with convalescent sera of bovine and human origin from cases other than leptospirosis (e.g. Q fever, syphilis). On the other hand, titers of at least 1:100 and up to 1:6400 were recorded in testing each of the antileptospiral sera. This indicates that the leptospiral LAT is specific in the sense that it detects only leptospiral antibodies, and does not reveal the presence of normal antibodies or specific antibodies against microorganisms other than leptospirae. It is interesting to note that, besides relatively much higher type-specific titers, intertype cross-reactions were also obtained in the lower dilutions of most sera, when tested with type-heterologous antigens.

Sensitivity of the Test

Comparative investigations were carried out on the sensitivity of the LAT as compared to that of the MAT and CFT. Rabbits were infected with live cultures of each of the three leptospira serotypes, respectively. Blood samples were drawn from the rabbits before infection and at various intervals after infection, and the sera tested with type-homologous antigens, to find out what differences could be detected in the sensitivity of the three serological tests employed as to the appearance, development, and disappearance of the type-specific antibodies.

Table VI shows the results obtained with sera of two rabbits infected with *L. canicola* cultures. One rabbit was bled at intervals of 2–3 days, and the other rabbit was bled weekly at first, later at monthly intervals after infection.

TABLE VI

Sensitivity of latex agglutination test

	Before infection	Days after infection: 2	4	7	9	11
		Titer of serum				
CFT	—	—	16	256	256	256
MAT	—*	—*	400	3200	6400	12,800
LAT	—*	—*	200	800	3200	3,200

	Before infection	Days after infection: 9	14	21	28	35	42	49	80	110	140	170	200
		Titer of serum											
CFT	—	512	1,024	1,024	512	256	128	64	64	32	32	8	—
MAT	—*	8000	16,000	16,000	16,000	8000	.	2000	1000	400	—*	—*	—*
LAT	—*	1600	6,400	6,400	6,400	3200	.	400	200	100	—*	—*	—*

NOTE: CFT = complement fixation test; MAT = macro-agglutination test; LAT = latex agglutination test; — = negative in the starting dilution of 1:8; —* = negative in the starting dilution of 1:100; . = not tested.

All three tests gave negative results up to the second day, but became positive in significant titers on the fourth day after infection. High titers were obtained with all three tests from the seventh to the ninth day on. The peak titers were detected by all three tests between the second and fourth weeks, then the titers decreased gradually. In about 4 months, neither the MAT nor the LAT gave positive results in a significant titer, while the CFT continued to be positive until about 6 months after infection.

Comparison of Different Serological Tests

Twenty-five leptospirosis-positive sera (among them 6 hyperimmune rabbit sera, 1 human and 18 bovine convalescent sera) were tested with the LAT and the most commonly used serological tests such as the MALT, MAT, and CFT.

In Table VII, the results obtained with only representatives of the various sera tested were included. The results given by the LAT compared favorably with those of the other three tests with some differences only in the magnitude of the titers and the degree of type-specificity. The highest titers were obtained with the MALT (up to 1:32,000), somewhat lower with the MAT (up to 1:16,000), still lower with the LAT (up to 1:6400), and lowest with the CFT (up to 1:256). Intertype cross-reactions were noted by all four serological tests, most consistently with the hyperimmune rabbit sera, but quite often with convalescent sera as well. The titers of intertype cross-reactions were relatively low as compared to the type-specific titer of a given serum, when the MALT or the MAT was used, the latter being at least 8–16 times higher than the former. Using the CFT or the LAT, the difference between the type-specific and group-specific titers was found to be less marked in some of the sera.

In connection with the use of the LAT for screening sera, it is of interest to note that the bovine convalescent sera tested with the LAT fell into four groups. Some sera (represented by serum E 2475) gave positive reactions

TABLE VII

Comparison of different serological tests

	Antigens	MALT	MAT	CFT	LAT
Rabbit hyperimmune sera					
C-587	C	16,000	12,800	256	6,400
	I	2,000	800	128	100
	P	1,000	−*	64	400
I-586	C	1,000	−*	8	100
	I	32,000	16,000	256	3,200
	P	4,000	1,600	16	200
P-586	C	−**	−*	64	400
	I	−**	100	64	800
	P	16,000	8,000	256	6,400
Human convalescent serum					
K-1614	C	6,400	3,200	64	3,200
	I	400	400	−	200
	P	400	200	−	100
Bovine convalescent sera					
E-2475	C	−**	−*	−	−*
	I	−**	−*	−	−*
	P	16,000	8,000	128	6,400
E-2480	C	−**	−*	16	200
	I	−**	−*	−	−*
	P	3,200	1,600	64	800
E-2483	C	−**	−*	32	−*
	I	400	−*	16	200
	P	12,800	1,600	128	1,600
E-2487	C	1,000	−*	64	100
	I	1,000	200	64	200
	P	16,000	6,400	256	1,600

NOTE: Titers are expressed in reciprocals. − = negative in a dilution of 1:8; −* = negative in a dilution of 1:100; −** = negative in a dilution of 1:400; C = *L. canicola*; I = *L. icterohaemorrhagiae*; P = *L. pomona*.

exclusively with *L. pomona* antigen. One serum (E 2480) showed intertype cross-reaction with *L. canicola* only, a few others (represented by E 2483) with *L. icterohaemorrhagiae* only, and the rest of the sera (represented by E 2487) cross-reacted with both type-heterologous test antigens. Nevertheless, each of these sera revealed four to eight times higher type-specific titers with *L. pomona* antigen. Similar results were obtained with the other three serological tests in screening the bovine convalescent sera. It should be mentioned, however, that the MALT and the MAT showed in general a somewhat higher type-specificity than the other two tests. On the other hand, the CFT yielded relatively stronger cross-reactions than the LAT.

Discussion

Among the various serological tests recommended for detection of leptospiral antibodies in human and animal sera, the MALT and different forms of the MAT are the most commonly used in diagnostic laboratories. Each has its merits as well as certain disadvantages. Due to its high specificity and sensitivity, the MALT is still the most widely used test, but requires the continuous maintenance of live cultures, an almost day-by-day care of seeding and harvesting leptospirae of different serotypes to have a set of suitable

antigens available at any time. In addition, microscopical reading of the MALT is laborious and time consuming. Different forms of the MAT (ordinary tube test, capillary tube test, slide or plate test) possess a similarly high degree of specificity and sensitivity with the additional advantage of being easily read by the naked eye. The MAT has, however, certain disadvantages. If living cultures are used, the difficulties mentioned above are encountered. Cultures killed by some means (most commonly by the addition of formalin) tend to lose their homogeneity on storage and to develop non-specific reactions.

The results of our studies on the leptospiral latex agglutination test indicate that it successfully combines the most important advantages of other leptospiral agglutination tests, and avoids some of their drawbacks. It possesses a high degree of specificity and sensitivity, which compares favorably with that of the MALT and MAT. It does not require the maintenance of live antigen supplies. The test antigens are not infective, since they contain no whole leptospiral organisms, do not clump spontaneously, and preserve their homogeneity even after prolonged storage. The test can be as easily and quickly performed as any common agglutination test even in laboratories equipped with the most modest facilities; macroscopical reading of the results is simple.

The CFT for leptospirosis as performed according to the method described previously (4) provides the same advantages as the LAT for laboratories, where other complement fixation tests are performed routinely. As shown by the results of our comparative investigations, both the CFT and the LAT can be used with equally good results for the serodiagnosis of leptospirosis. The persistence in the sera of antibodies detectable by the CFT for a longer period than by the LAT should be mentioned in the favor of the former. On the other hand, the LAT has the advantage of a somewhat higher type-specificity than the CFT. Other factors such as the frequency of using the CFT in a given laboratory, and availability of trained personnel, equipment, etc., may also contribute to the decision as to which one of the two tests should be preferred in screening sera for leptospirosis.

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NOTES

PRESERVATION OF XANTHOMONAS BY FREEZING IN GLYCEROL BROTH[1]

C. QUADLING

The efficacy of freezing as a means of preserving bacteria varies with species and with suspending medium; sugars and glycerol are amongst the best-known agents for reducing mortality due to freezing (10). There are a number of reports (1, 2, 4, 11) of the preservation of bacteria at low temperatures in media containing 10% to 15% glycerol. Routine storage of stock cultures in the frozen condition has obvious advantages; for example, the possibility of population changes due to mutation and selection during storage is eliminated. For this and other reasons, quantitative aspects of the survival of frozen *Xanthomonas phaseoli* (XP8) cells were studied and the results are reported here.

The methods used have been described (8). In addition, shake cultures of *X. phaseoli* (XP8-1) were grown in Difco Antibiotic Medium No. 3 (Penassay broth) to a viable cell density of about 10^9/ml in the late logarithmic phase. Cultures were diluted by addition of an equal volume of sterile broth containing 30% v/v glycerol; the final concentration of glycerol was therefore 15%. Aliquots (1 ml or 10 ml) of this suspension were distributed into sterile screw-capped vials and frozen by being placed in a freezer operating at −20° C. When required, stored cultures were thawed in air at room temperature (ca. 25° C). Cells stored in this way for less than 4 weeks will be referred to as "short-term" and those held at −20° C for longer periods as "long-term" cultures.

To determine the effects of such treatment on viability of *X. phaseoli*, colony counts (6, 8) were made on aliquots of a culture before and after admixture with glycerol broth and after various periods of storage at −20° C. The results in Table I show that most bacteria survived mixture with glycerol and subsequent freezing and thawing. An increasing proportion of bacteria failed to form colonies with increasing length of storage. No difference was found among plate counts made from short-term culture aliquots thawed by standing in air at 4° C, in air at room temperature, or in a water bath at room temperature. Refreezing in the absence of glycerol resulted in a big drop (Table I) in viable count. Although XP8-1 is prototrophic, fewer cells formed colonies on a simple glucose–salts agar medium after long-term storage than on Tryptone or Penassay agar. Straka and Stokes (9) reported similar observations with *Pseudomonas* and *Escherichia*; they suggested that such variations in colony-forming ability resulted from metabolic injury received at low temperatures and repaired by complex nutrients.

The effects of storage at −20° C on subsequent growth of cultures was investigated by thawing 10-ml aliquots of dense suspensions of cells, diluting

[1]Contribution No. 498, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa, Canada.

TABLE I

Effect of storage at −20° C, and of subsequent treatment,* on colony-forming ability of frozen aliquots of a culture of *X. phaseoli*

Storage (days)	Viable count† $\times 10^{-8}$/ml	Remarks	Storage (days)	Viable count† $\times 10^{-8}$/ml	Remarks
	26.0	Initial count	301	3.0	
	10.0	After dilution 1:1 with glycerol broth	314	2.7	
1	10.8		314	3.3	Proteose–tryptone–agar
7	9.5		314	0.36	Salts–glucose–agar
14	8.4		405	1.40	
28	6.2		466	0.40	
42	6.4		466	0.37	Thawed and refrozen six times
133	2.6		466	0.0002	Refrozen in nutrient broth without glycerol

*All platings on Difco antibiotic medium 2 unless otherwise stated. All dilutions in antibiotic medium 3 broth or in liquid equivalent of stated solid medium.
†Stated results are means of plate counts made on two or more replicate culture-aliquots.

these one in five with fresh broth, and incubating with agitation at 26° C. Turbidity measurements were made at intervals. Cultures stored for short periods (less than 4 weeks) resumed logarithmic growth with a lag, if any, of less than 30 minutes. After prolonged storage (6 months) there was an initial drop in turbidity, presumably due to lysis, and a lag of 4 to 5 hours before logarithmic growth was resumed. In the latter case, plate counts made in parallel with turbidity measurements showed that the number of viable cells remained constant during the lag period and that cell multiplication resumed when the turbidity started to increase.

To investigate the possibility of mutagenic effects on stored bacteria as a result of exposure to low temperatures, the rate of mutation from the streptomycin-sensitive to the streptomycin-indifferent condition was determined. Mutagenic effects occurring during low temperature storage have been reported in *Streptomyces* (12). Evidence for the occurrence of heritable modifications in tissue cultures following low temperature storage has been reported (7) and subsequently explained (3) as a predominantly selective effect. The rate of mutation in cultures of XP8-1 was determined by a modification of the "*Po*" method of Luria and Delbrück (5) as used in a previous study (8). Inocula for about 50 replicate flask cultures were derived from frozen short-term or from long-term cultures of XP8-1 by thawing and subsequent dilution in fresh nutrient broth. These cultures grew for about 10 mean generations prior to challenge with streptomycin at a final concentration of 10^3 μg/ml, when about 10^9 sensitive cells were present in each replicate culture. Mutation rates were calculated from the proportion of cultures without mutants and the mean viable count at the time of challenge. The mean value for mutation rate found in three such experiments was 2.96×10^{-10} per bacterium per generation. This is very similar to the mean value of 3.24×10^{-10} previously reported (8) for *Xanthomonas*. It is concluded that no immediate or delayed mutagenic effects of cold storage on the streptomycin character had occurred.

Stock cultures of a number of phytopathogenic and related bacteria have been preserved satisfactorily as described above and also by picking single colonies directly into small volumes of glycerol broth. Such stock cultures include *Rhizobium*, *Erwinia*, and *Pseudomonas* species, in addition to a number of different *Xanthomonas* sp. and mutant stocks of *X. phaseoli*. No deleterious effects of such storage on genetic stability, on virulence, or on lysogenicity (where applicable) have been noted; however, the only quantitative observations made were those reported above.

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MICROBIOLOGY RESEARCH INSTITUTE,
RESEARCH BRANCH,
CANADA DEPARTMENT OF AGRICULTURE,
OTTAWA, ONTARIO.

ISOLATION OF BACTERIOPHAGE FOR ARTHROBACTER GLOBIFORMIS[1]

D. C. GILLESPIE

Although bacteriophage isolations have been reported for members of various genera of organisms found in soil, such as *Mycobacterium* (1), *Corynebacterium* (2), and *Streptomyces* (3), no phage isolations have been reported for members of the genus *Arthrobacter*, although Robinson and Corke (3) observed plaques when soil perfusates were plated with *Arthrobacter*.

[1]Contribution No. 501, from the Microbiology Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.

Arthrobacter globiformis strain 616 was inoculated into fertile field soil which was moistened with mineral medium (4) containing 1.0% sucrose and 0.2% vitamin-free casamino acids. The soil was incubated 2 days at room temperature, triturated with water, and the centrifuged extract passed through a UF grade sintered glass filter. The filtrate was diluted with mineral – sucrose – amino acid broth and mixed with exponentially growing cells of *A. globiformis*. After allowing 15 minutes for absorption, 1.0 ml of the various dilutions was plated on the dry surface of the above medium containing 1.5% agar.

Two bacteriophage isolates were obtained. One designated 616 L produced plaques about 1.5 mm diameter; the other, 616 S, produced plaques 0.5 mm diameter.

Lysates containing 5×10^9 to 8×10^9 infectious particles per milliliter were readily obtained when early exponential phase cultures were inoculated with either virus. Both bacteriophage strains were stable on storage. Lysates of 616 L after 4 months' storage at 4° C dropped from 8×10^9 to 1.3×10^9 plaque-forming units per milliliter. A 616 S lysate in the same period remained at 7.6×10^9 units per milliliter.

The plaque count of 616 S when plated with *A. globiformis* strain 425 was the same as when it was plated with strain 616. The large plaque virus, however, had a titer of less than 10^5 on strain 425; it will lyse this strain in fluid culture when 10^8 units are inoculated in early exponential phase cultures. Further cross infectivity tests were made using 10 *Arthrobacter* species from the Institute collection. The original soil filtrate, as well as 616 S, 616 L, and a supernatant filtrate from a supposed lysogenic culture of *A. globiformis* 616, showed no evidence of lytic action either in plate or in broth culture.

A colony arising in the center of a 616 L plaque was subcultured and several single colony isolations made. This isolate is resistant to lysis by both 616 S and 616 L bacteriophage strains. While an extensive study has not been made, this culture appeared to be lysogenic. Titers of supernatant culture fluid of this clone were quite high, yielding plaque counts of 1.6×10^8 when plated with either strain of *A. globiformis*.

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